Inactivation of a human kinetochore by specific targeting of chromatin modifiers

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I hereby certify that all the work presented in this thesis is my own except where stated otherwise, and that it has been entirely composed by myself.

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ABSTRACT

Here I describe the construction and characterization of a new generation of human artificial chromosome that contains an array of DNA sequences that can be used to manipulate the chromosome *in vivo* and possibly *in vitro*. This HAC was originated in human fibrosarcoma HT1080 cells from a synthetic alphoid DNA containing an array of TetOperator sequences, cloned in a BAC-based vector. This synthetic α-satellite DNA formed HACs that were stably maintained throughtout replication and segregation in HT1080 cells. However, I succeeded to also transfer and manipulate the alphoid^{tetO} HAC into a HeLa-based hybrid cell line. The synthetic alphoid^{tetO} HAC chromatin was similar to the chromatin at endogenous centromeric alphoid DNA. Importantly, the DNA sequences embedded in the synthetic HAC were accessible to targeting TetR-fused constructs *in vivo*.

The alphoid^{tetO} HAC could be successfully targeted with a number of TetR:fusion proteins without affecting its chromatin structure, kinetochore assembly and mitotic behaviour. However, the targeting of a transcriptional activator (tTA) inactivated the HAC synthetic alphoid^{tetO} DNA in a fraction of transfected cells. Surprisingly, the targeting of the transcriptional repressor tTS, co-repressor KAP1 or the heterochromatin-associated protein HPIα severely inactivated the synthetic alphoid^{tetO} kinetochore . In fact, upon targeting several inner and outer kinetochore proteins were delocalized from the alphoid^{tetO} sequences. The dissociation of kinetochore proteins CENP-H and CENP-C appeared to precede that of CENP-A. The alphoid^{tetO} HAC lacking inner kinetochore protein complexes showed mitotic defects including misalignment at the metaphase plate and defective anaphase segregation, ultimately being included in tiny DAPI-positive nano-nuclei in the cytoplasm.

The transcriptional repressor tTS repressed the low levels of transcription from the alphoid^{tetO} sequences. In addition, targeting of transcriptional repressors altered the HAC chromatin towards a more “closed”, heterochromatic conformation, as seen from the changes in histone tail modifications. Interestingly, the targeting of the histone methyltransferase

EZH2 to the alphoid^{teto} HAC showed a much milder inactivating activity compared to KAP1.

Based on these results, I propose that the formation of HPI-type of heterochromatin or accumulation of HPIα to the centromeric regions could disrupt the association of constitutive kinetochore proteins to the underlying sequences. Centromeric alphoid sequences lacking a functional kinetochore structure then also loose the centromere-specific histone H3 variant CENP-A becoming definitively inactive. Alternatively, a basal transcriptional activity from centromeric sequences might be required for centromere functionality.

I.Introduction

1. Chromosomes and mitosis

The genetic information in eukaryotic cells is encoded in the DNA, which is itself complexed with histones to form the material we call chromatin. The fundamental unit of the chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around an octamer of two of each of the highly conserved histones H2A, H2B, H3 and H4 (Luger et al., 1997; reviewed in Wolffe and Kurumizaka, 1998). At a higher level of organization, nucleosomes are structured into a 10 nm fibre, which is then further compacted into a 30 nm chromatin fibre, facilitating the packing of DNA into the nucleus (reviewed by (Robinson and Rhodes, 2006).

During cell division, chromatin is condensed into clearly discernable discrete entities called chromosomes. The organization of the replicated DNA into pairs of sister chromatids, which are pulled towards opposite spindle poles during mitosis, facilitates the equal distribution of the genetic material between two daughter nuclei. In fact, the maintenance of genome integrity is crucially dependent on accurate segregation of chromosomes in mitosis and meiosis, but it is not yet fully understood exactly how the cell completes this regulated process. Mistakes that result in the gain or loss of chromosomes (aneuploidy), or the formation of chromosome aberrations, are associated with diseases that may lead to death in humans (reviewed by Cahill et al., 1998). For example, in somatic cells chromosome instability and aneuploidy has been found to be associated with tumour development (reviewed by Sen et al., 2000).

Mitosis is divided into six stages, which are illustrated in Figure 1: prophase, prometaphase, metaphase, anaphase (can be divided in Anaphase A and B), telophase, and cytokinesis. In **prophase**, the chromatin starts to condense through a process that ultimately leads to the formation of distinct chromosomes, consisting of paired sister chromatids as mentioned above. The centrosomes, which are the main microtubule organizing structure in animal somatic cells, are duplicated beginning during S phase in

a process that takes more than an entire cell cycle to complete. During prophase, they separate, moving across the surface of the nucleus.

The transition to **prometaphase** is marked by the breakdown of the nuclear envelope, which consists of two lipid bilayers (the inner and outer membranes) associated with a proteinaceous lamina enclosing the nucleus. After the breakdown of the nuclear envelope, the condensed chromosomes are exposed to the cytoplasm, where the mitotic spindle is starting to assemble. Microtubules emanating from the spindle poles, formed at the separated centrosomes, stretch in every direction, eventually stochastically encountering kinetochores, the protein structures involved in connecting each sister chromatid to the spindle. The chromosomes, through dynamic interactions with the spindle microtubules, gradually move towards the spindle equator.

Chromosomes that have aligned at the spindle equator are said to have congressed at the metaphase plate. When all chromosomes are aligned, the cell reaches **metaphase.** At this stage, each pair of sister chromatids are connected to opposite poles, a state referred to as bi-orientation.

In **anaphase**, sister chromatids separate, moving towards the spindle poles. In **anaphase A,** they separate and begin this movement, but the spindle poles themselves maintain their distance. Shortly afterwards, in **anaphase B,** the spindle elongates and the distance between the poles increases.

The nuclear envelope begins to form around the chromosomes during **telophase**, and only when this is complete do the chromosomes decondense and form the new daughter nucleus. During late anaphase B and telophase, a cleavage furrow is formed between the nuclei and begins contracting towards the central spindle, or mid-zone.

In **cytokinesis,** this contraction has reduced the cytoplasm between the two daughter cells to a thin intercellular bridge containing a dense array of microtubules, called the mid-body. This structure eventually splits and the two daughter cells separate.

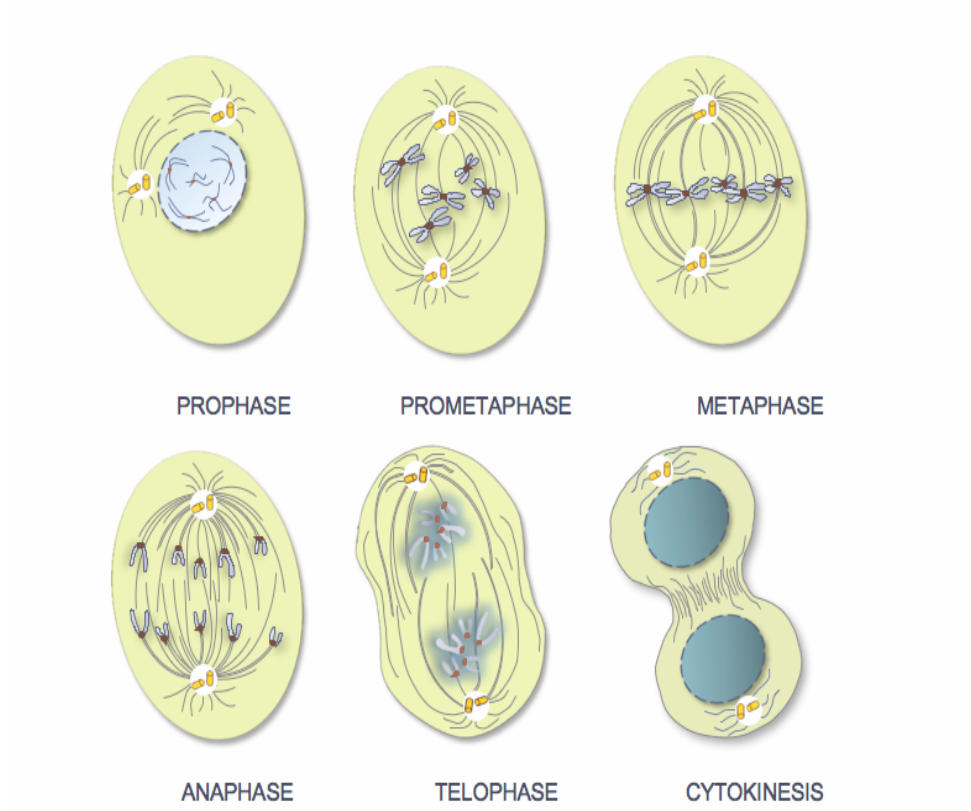


Figure 1. Stages of mitosis.

2. The centromere

The centromere is a chromosomal region that comprises the primary constriction, which is readily visible in the condensed mitotic chromosomes of higher eukaryotes. Although the vast majority of the chromosomal DNA is pulled apart passively during the process of mitotic chromosome segregation, the centromere plays an active and essential role in this process. The centromere is the site of sister-chromatid cohesion and senses the tension that each replicated chromosome is subject to when it is attached to mitotic microtubules emanating from the opposite spindle poles (that is, when it is bioriented). In fact, the centromere defines the location of the kinetochore, a proteinaceous structure that assembles on the centromere surface at each cell division and mediates the attachment of the spindle microtubules (see below). One and only one centromere must function on every chromosome at anaphase to avoid chromosome loss or breakage. Furthermore, the exact position of the centromere is accurately inherited from one cell lineage to the next. Despite the central role of the centromere in some of the most important mechanisms in eukaryotic cell division, its underlying structure and specification are still poorly understood.

2.1 DNA sequence as centromere determinant

The discovery of sequence-specific “point” centromeres in the budding yeast *Saccharomyces cerevisiae* supported a model of sequence-based centromere positioning which was thought to be valid also in metazoans (Clarke and Carbon, 1980). However, many years of study have failed to identify sequence elements sufficient to define centromeric regions in higher eukaryotes.

Centromeres that have been characterized range in size from the 125 bp centromere of budding yeast to centromeres of several megabases in human chromosomes. In contrast to budding yeast, the centromeres of animals and plants (and also the fission yeast *Schizosaccharomyces pombe*) are typically comprised of highly repetitive satellite DNA. Human centromeric DNA

consists of arrays of an α-satellite (with a monomer consensus sequence of 171 bp) which is repeated head-to-tail over a region spanning several megabases at most centromeres (Willard et al., 1987). This monomer consensus sequence is clustered in higher order repeats that constitute Type I alphoid DNA and form the core of centromeres. Type II alphoid DNA, which usually flanks Type I arrays, is characterized by a less regular arrangement of monomers (Ikeno et al., 1994).

An important element of satellite DNA is a 17 bp sequence known as the CENP-B box, which is the DNA binding site for the centromeric protein CENP-B (Masumoto H et al., 1989). CENP-B boxes are highly enriched in Type I alphoid DNA, but not in Type II arrays (Ikeno et al., 1994).

Several studies have failed to ascertain a fixed link between centromeric DNA and kinetochore assembly. Furthermore, it has not been possible to identify an unambiguous sequence element shared among centromeres. Centromereic DNA sequences are highly variable across the phylogeny with no obvious conservation even among closely related species. This suggests that centromeres are probably specified by epigenetic mechanisms (reviewed in Sullivan KF., 2001).

2.2 Dicentric and neocentromeric chromosomes

Much insight regarding the role of the DNA sequence in defining centromere location has come from the discovery and characterization of dicentric and neo-centromeric chromosomes.

In a dicentric chromosome, complex duplication events or rearrangements give rise to two regions containing sequences normally found at the centromere (Earnshaw and Migeon, 1985; Earnshaw et al., 1989). In the case of stable autosomal dicentric chromosomes, one of the two centromeres is inactivated (centromere inactivation) and does not recruit a functional kinetochore structure (Figure 2) (Earnshaw and Rothfield, 1985; Earnshaw et al., 1989; Sullivan and Schwartz, 1995; Warburton and Cooke, 1997). However, both regions bind the centromeric protein CENP-B, suggesting that association of CENP-B is not sufficient to activate the centromere for

kinetochore assembly. CENP-B is also apparently not necessary for centromere function (Figure 2), because it is not associated with the alphoid region of chromosome Y (Haaf et al., 1995), and because CENP-B knockout mouse cells are capable of assembling functional kinetochores (Hudson et al., 1998; Perez-Castro et al., 1998; Kapoor et al, 1998).

Neo-centromeric chromosomes are also the product of complex re-arrangements, but in this case a kinetochore is found to assemble at non-centromeric DNA. Neo-centromeric chromosomes have been found in both flies and humans (Murphy and Karpen, 1995; Byron C Williams et al, Nature 1998). In Drosophila, structurally acentric mini-chromosomes deleted of all centromeric sequences acquired centromeric activity and showed properties associated with chromosome inheritance (Murphy and Karpen, 1995; Voullaire et al., 1993). In this study, the same sequences separated from the natural centromere failed to show centromeric activity. This suggests that the DNA sequence alone is not sufficient to trigger *de novo* centromere formation, but possibly has to be somehow "activated".

Neocentromeres have also been discovered in humans. The best characterized of these structures was found in a marker chromosome following a complex rearrangement of normal chromosome 10 (mardel10) (Voullaire et al., 1993; du Sart et al., 1997). This neocentromere was shown to have originated from a non-rearranged euchromatic region, with no remarkable structure or DNA content (Barry et al., 1999). Detailed analyses to identify a sequence determinant for the incorporation of CENP-A, an important centromere marker thought to designate centromere identity, have been inconclusive. The AT-content (a DNA sequence feature commonly found to be enriched at centromeres) of CENP-A and non-CENP-A-binding clusters of the neocentromere were similar (Chueh et al., 2005). When interspersed repetitive DNA elements were analyzed, only the long interspersed nucleotide element (L1) was found to be a possible, although weak, candidate for a CENP-A binding target sequence (Chueh et al., 2005). DNA sequence comparison between the mardel10 neocentromere and the

progenitor allele from which it was derived demonstrated that they were identical in sequence (Barry et al., 2000).

In summary, these studies have so far failed to identify any sequence element that is necessary and sufficient for centromere establishment.

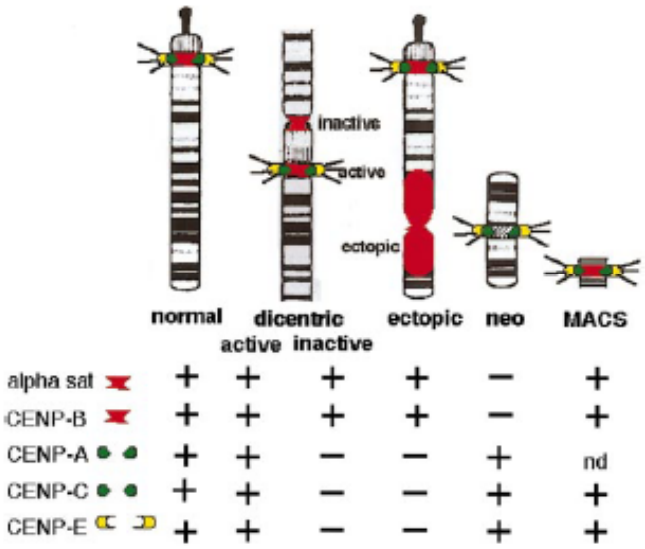


Figure 2. Localization of CENP-proteins and α-satellite DNA to active and inactive centromeres. CENP-A and CENP-C, but not CENP-B, localize only to active centromeres and neocentromeres. A functional kinetochore can assemble also on neocentromeres lacking of α-satellite DNA**.**

2.3 Human Artificial Chromosomes

Important findings about the factors required for the establishment of centromeric activity, and mitotic stability, have come from investigating the de novo formation of artificial chromosomes.

Pioneering studies of the functional elements required for constructing artificial chromosomes were first done in *S. cerevisiae* and later in *S. pombe* yeast species. These studies of Yeast Artificial Chromosomes (YACs) determined that at least three types of chromosomal elements are required to produce a linear, mitotically stable artificial chromosome – centromeres, telomeres and origins of replication (Clarke and Carbon, 1980; Murray and

Szostak, 1983). In mammalian cells, artificial chromosome technology was hindered for a long time, in part because of the more complex structure of chromosomes and centromeres, but also because it was not possible to produce large, stable fragments of highly repetitive centromeric DNA (Neil et al., 1990). The development of technologies for cloning lengthy alpha satellite arrays, such as single copy-number plasmids (Harrington et al., 1997) or YAC-based vectors in recombination-deficient yeast strains (Ikeno et al., 1998), allowed the construction of the first generations of Human Artificial Chromosomes (HACs) (Figure 2).

The first minichromosomes obtained were mostly a product of chromosome truncation events (Harrington et al., 1997), but it was eventually possible in human HT1080 cells to obtain, quite efficiently, HACs that were originated by a de novo mechanism and did not acquire detectable host sequences (Ikeno et al., 1998). This work also provided landmark evidence identifying elements required for HAC construction. For example, only Type I, but not Type II, alpha satellite DNA was able to efficiently form stable HACs (Ikeno et al., 1998). Since CENP-B binding sequences appear only in Type I alphoid DNA, it seemed that, in contrast with data from neo-centromeric chromosomes (Saffery et al., 2000), CENP-B binding had a role in the de novo formation of centromeric activity (Figure 2). Confirming these observations, chromosome 21 Type I alphoid DNA with a mutated CENP-B box was unable to form HACs, and all input DNAs were integrated into host chromosomes in cells carrying the drug resistance marker (Ohzeki et al., 2002). However, if CENP-B boxes were inserted into exogenous GC-rich non-alphoid DNA, they would not confer HAC formation ability. Thus, neither Type I alphoid DNA nor CENP-B boxes were sufficient to promote *de novo* kinetochore assembly. This suggested that there must be an “epigenetic” element in Type I alphoid DNA, other than the CENP-B box, which is important for de novo centromere formation (Ohzeki et al., 2002).

The epigenetic character of centromeres has been well described. Thus far, it appears that in addition to sequence elements, centromeres are specified by conformational or structural components at the chromatin level.

3. The assembly of centromeric chromatin

3.1 CENP-A marks the centromere

CENP-A is a centromere-specific histone variant specifically found in centromeric nucleosomes (Palmer et al., 1991; Sullivan et al., 1994). CENP-A homologues are found in all eukaryotes and are tightly associated with kinetochore activity. This conserved histone H3 variant localizes at all active centromeres (Earnshaw and Migeon, 1985), as well as natural or functional variants of centromeres. In stable dicentric chromosomes, CENP-A is absent from the inactivated centromere but it is found at the active neocentromere that lacks α-satellite repeats (Warburton et al., 1997; Amor et al., 2004). This localization at active versus inactive centromeres makes CENP-A the most attractive candidate for an epigenetic mark of centromere function. In fact, RNAi depletion of CENP-A in human cells and a genetic knockout in chicken DT40 cells have clearly shown that CENP-A is necessary for the maintenance of centromeric activity (Goshima et al., 2003; Regnier et al., 2005).

Human CENP-A shares approximately 57% identity with histone H3. This homology is restricted to the C terminal portion. The CENP-A N-terminus is also highly divergent among species (Sullivan et al., 1994). *In vitro,* nucleosome reconstitution shows that CENP-A is able to co-assemble with histones H2A, H2B and H4 into octameric nucleosomes, replacing both copies of histone H3 (Shelby et al., 1997; Yoda et al., 2000). Consistent with the high level of homology found throughout the C terminus of CENP-A in different species, it is this globular region of the protein that determines its targeting to the centromere (Sullivan et al., 1994).

The mechanism by which CENP-A marks the centromere location is still poorly understood. One possibility is that the presence of CENP-A alone determines the location of the kinetochore. However, in human tissue culture

cells, when CENP-A was over-expressed and incorporated into non-centromeric regions, it could recruit the inner kinetochore protein CENP-C but it could not produce functional ectopic kinetochores (Van Hooser et al., 2001). Recently, over-expression of CENP-A^{CID} in both developing flies and fly tissue culture cells was shown to induce the formation of kinetochore-like structures at ectopic loci that were possibly capable of making microtubule attachments (Heun et al., 2006).

Another possibility is that CENP-A nucleosomes are structurally different from bulk nucleosomes and confer a special conformation on centromeric chromatin. For example, *in vitro* studies have suggested that CENP-A nucleosomes are more conformationally constrained within the α2-helix of CENP-A and the α2- and α3-helices of histone H4 (Black et al., 2004). Twelve of the CENP-A α2-helix amino acid residues, together with the preceding loop (L1), differ from the corresponding positions in canonical H3 and form the Centromere-Associated Targeting Domain (CATD). Substitution of the CATD into canonical histone H3 is sufficient to confer upon it the essential centromere maintenance activity of CENP-A (Black et al., 2007b). Since the CATD is sufficient to confer structural rigidity to the nucleosomes, it has been proposed that this CATD-mediated rigidity provides the targeting information for newly synthesised CENP-A as well as the nucleosomal mark for its deposition (Black et al., 2007a).

In Drosophila, CENP-A-containing nucleosomes prepared from interphase cells were reported to be composed of heterotypic tetramers (i.e. CENP-A, H4, H2A and H2B), instead of typical octamers (Dalal et al., 2007). It has therefore been suggested that CENP-A nucleosomes might be half the size of normal nucleosomes as well as being more rigid. However, in another study a tagged-version of CENP-A, introduced into HeLa cells, isolated mostly homotypic octameric nucleosomes (i.e lack H3), and a small pool (~10%) of heterotypic nucleosomes (Foltz et al., 2006).

Regardless of the composition of CENP-A nucleosomes, which is still controversial, these nucleosomes may be responsible for a compact structure that is essential for the structure and function of centromere chromatin. One attractive idea is that exclusion of H3-nucleosomes from the centromere is mediated by the structural divergence and rigidity of the CENP-A nucleosomes.

3.2 Timing CENP-A recruitment

Initially centromere DNA sequences were thought to be late replicating, which suggested that the time of replication might be important in propagating CENP-A chromatin at centromeres (Csink and Henikoff, 1998). However, it was later shown that centromeres replicate asynchronously and that there is no time in S-phase when only centromeres are being replicated (Shelby et al., 2000; Ahmad and Henikoff, 2001). This suggested that replication timing is unlikely the trigger for incorporation of CENP-A into centromeric DNA.

The fact that CENP-A assembly can take place in the presence of the replication inhibitor aphidicolin suggested that incorporation of CENP-A into centromeric nucleosomes is probably uncoupled from replication (Shelby et al., 2000). In fact, although DNA is replicated in S-phase, human CENP-A is not expressed until G2 (Shelby et al., 2000). In animal cells, newly synthesised CENP-A is incorporated into centromeres only starting from late mitosis and continuing well into the next G1 phase (Jansen et al., 2007; Schuh et al., 2007), reviewed in (Black and Bassett, 2008). These investigations suggest that during DNA replication in S phase, CENP-A is randomly distributed between the two DNA strands, and chromosomes face mitotic segregation having only half of the CENP-A binding sites occupied in centromeres. An epigenetic ‘priming’ of the nucleosomes, where CENP-A has to be recruited, might be required for the faithful transmission of centromere identity from one cell cycle to the next.

3.3 Marking the site of CENP-A loading

The nature of the priming event for CENP-A loading into centromeric nucleosomes is still being actively investigated (Henikoff and Dalal, 2005). The temporal loading of CENP-A into centromeres is mediated, at least in part, by proteins Mis18 and KNL2 (also called Mis18 Binding Protein 1, M18BP1) (Fujita et al, 2007). In human cells, hMis18 isoforms α and β, together with hKNL2, localize at centromeres in the same time window of CENP-A (Fujita et al., 2007; Maddox et al., 2007). A possible indication of how hMis18α/β may function comes from the finding that CENP-A mis-incorporation caused by depletion of hMis18, can be rescued by treatment with the histone deacetylase inhibitor Trichostatin A (Fujita et al., 2007). Human hMis18 isoforms have been found to be associated with RbAp46/48, which are highly similar to the fission yeast Mis16. Also, the RbAp46/48 proteins have been shown to be necessary for the recruitment of CENP-A, because in cells where both are depleted, virtually all kinetochore CENP-A signals are abolished (Hayashi et al., 2004). In fission yeast, the Mis16-Mis18 complex is essential for localizing CENP-A to centromeres (Hayashi et al., 2004). RbAp46/48 proteins are part of the Chromatin Assembly Factor 1 (CAF1), which is implicated in the deposition of H3/H4 dimers during DNA replication. Intriguingly, the CAF-1 complex co-purifies only with the replication-dependent histone variant H3.1, but not with H3.3 or CENP-A-containing nucleosomes (Foltz et al., 2006). Thus, the assembly factor for CENP-A is not yet known.

In conclusion, specifically modified H3 histones may play a crucial role in marking where CENP-A is loaded. One possibility is that in S phase histones H3 are placed at the 'CENP-A sites', and only later exchanged with CENP-A (Sullivan, 2001); reviewed in (Henikoff and Dalal, 2005; Vagnarelli and Ribeiru, 2008). However, to date there is no direct evidence that histone H3 is removed and replaced by CENP-A. Another model proposes that priming

for CENP-A loading involves a specific modification, such as deacetylation, of neighbouring H3-containing nucleosomes, rather than the nucleosomes where CENP-A is incorporated (reviewed in Black and Bassett, 2008).

3.4 Transcription and CENP-A loading

There is increasing evidence that centromeric sequences are transcribed and that this contributes to centromeric function. Recent analyses of human neocentromeres, and studies of the CENP-A binding region in rice, showed that centromeres can contain active genes and that they are competent for transcription (Saffery et al., 2003; Nagaki ., 2004; Yan et al., 2006). Recently, it was also shown that murine minor satellite repeats at the centromere accumulate transcripts ranging from 50 to 100 nucleotides under conditions of stress and differentiation (Bouzinba-Segard et al., 2006). A functional role for these transcripts was observed by forcing expression of a 120 nucleotide non-coding RNA. This caused misalignment of chromosomes in mitosis and defects in sister chromatid cohesion (Bouzinba-Segard et al., 2006).

Interestingly, studies in human, flies and fission yeast have shown that CENP-A nucleosomes at the centromere are interspersed with H3 nucleosomes that are dimethylated at K4 (H3K4me2) (Sullivan and Karpen, 2004; Cam et al., 2005; Lam et al., 2006), which is a mark of 'transcriptionally competent' chromatin. However, not all centromeres possess detectable amounts of this modification (see Vagnarelli et al., 2008). In mouse and human cells, H3K9me3 chromatin (usually associated with transcriptionally silent loci) is present at the centromeric satellite DNA together with CENP-A (Guenatri et al., 2004; Nakashima et al., 2005; Lam et al., 2006).

The functional significance of the differently modified H3-nucleosome subsets at the centromere is still obscure. One model places H3K4me2-

modified histones, surrounding unmodified nucleosomes, into gaps left by CENP-A after DNA replication in S-phase (Sullivan and Karpen, 2004; reviewed in Schueler and Sullivan, 2006). This process could establish the framework for the subsequent exchange of H3 histones with CENP-A. In contrast, a balance between the coexisting CENP-A and H3K9me3 nucleosomes appears to be crucial in determining the functional centromere structure (Okada et al., 2007). CENP-B may play a dual role in modulating these two antagonistic substrates. In fact, CENP-B not only induces CENP-A assembly on episomal (non-integrated) alpha-satellite DNA, but also enhances the competing and incompatible H3K9me3 modification on the same sequences integrated into a host chromosome (Okada et al., 2007). Thus CENP-B can both promote and inhibit kinetochore formation.

It is still not clear what level of chromatin conformation and transcriptional competency is necessary for a functional centromere. The fact that CENP-A and specially modified histone H3 are found at centromeres suggests that distinct combinations of CENP-A and H3 nucleosomes may signify a particular type of chromatin – centrochromatin – which specifies the position for kinetochore assembly on chromosomes (reviewed in Dunleavy et al., 2005).

3.5 Role for the kinetochore in CENP-A loading

Initial investigation on the fission yeast kinetochore component Mis6 revealed that kinetochore proteins may be implicated in loading CENP-A onto the underlying chromatin. In fact, in *mis6* mutants the localization of CENP-A^{Cnp1} to the centromere is greatly diminished. Also, Mis6 is required for the incorporation of newly synthesised CENP-A^{Cnp1}-GFP (Takahashi et al., 2000). Moreover, Mis6 is found in complex with Sim4 and *sim4* mutants also show reduction in CENP-A^{Cnp1} association with the centromere (Pidoux et al., 2003). The vertebrate homologue of Mis6 is CENP-I, which localizes to the inner kinetochore in a complex with the Sim4-related protein CENP-H

(see below). Initial studies on interference with CENP-H/I failed to identify any effect on centromeric CENP-A recruitment (Goshima et al., 2003; Nishihashi et al., 2002). However, a more recent study has shown that depletion of the CENP-H/I complex in chicken DT40 cells results in failure to incorporate newly synthesised CENP-A (Okada et al., 2006). In addition, in Drosophila S2 cells CENP-A, CENP-C and the previously uncharacterized protein Cal1 are mutually dependent for the localization at the centromere, because RNAi depletion of any single protein disrupted or diminished the localization of the other two (Goshima et al, 2007).

A further line of investigation in *S. pombe* revealed that a pre-kinetochore structure may be maintained throughout replication and that a single kinetochore can give rise to two functional daughter kinetochores (Hayashi et al., 2004). This process does not seem to require additional components that are not already part of the kinetochore itself. This result suggests that the presence of a pre-kinetochore structure at the onset of mitosis might be sufficient for positioning the new assembly of CENP-A.

In HeLa cells, overexpression of CENP-A and CENP-C resulted in their mis-targeting to non-centromeric sites along the chromosome arms (Van Hooser et al., 2001). However, centromere activity was not detected to these ectopic sites, even when the expression of additional CENP-A allowed its deposition at inactive centromeres on dicentric chromosomes. These results suggested that events at active centromeres such as the attachment of microtubules to the kinetochore or the tension generated between sister kinetochores may provide additional “marks” for centromere inheritance (Mellone and Allshire, 2003). In particular, the recruitment of newly synthesized CENP-A from mitotic telophase supports this model.

4. Kinetochore

4.1 Kinetochore structure

The kinetochore is assembled from several protein complexes at the centromere of each chromatid in a replicated chromosome. This structure is of paramount importance for mitotic chromosome segregation, because it is responsible for microtubule attachment and thus chromosome movement. Electron microscopy studies of the structure of mammalian kinetochores have revealed a trilaminar organization in which two electron-dense plates are separated by an electron-translucent middle region (Figure 3) (Jokelainen, 1967; Brinkley et al, 1966; Comings and Okada, 1971). These studies also showed that the trilaminar structure is only visible from late prophase until the end of mitosis, suggesting that the kinetochore undergoes cycles of assembly/disassembly (Roos UP, 1973; Brenner et al., 1981). Several recent mass-spectrometry studies have identified more than 100 proteins that are associated, at some stage, with the centromere/kinetochore structures in human cells (Foltz et al., 2006 Okada et al., 2006). Some of these proteins are found constitutively at the centromere (or CCAN, see below), and presumably take part in the earliest steps of kinetochore assembly during G2-phase, when they become part of the inner kinetochore plate. Other proteins show a temporal order of appearance at the kinetochore, starting in late G2-phase. Some of these proteins are involved in the important interface between kinetochore and microtubules (or KMN, see below), while others some are motor or regulatory proteins that take part in the fine-tuning of microtubule attachment and chromosome movement (reviewed in Fukagawa, 2008).

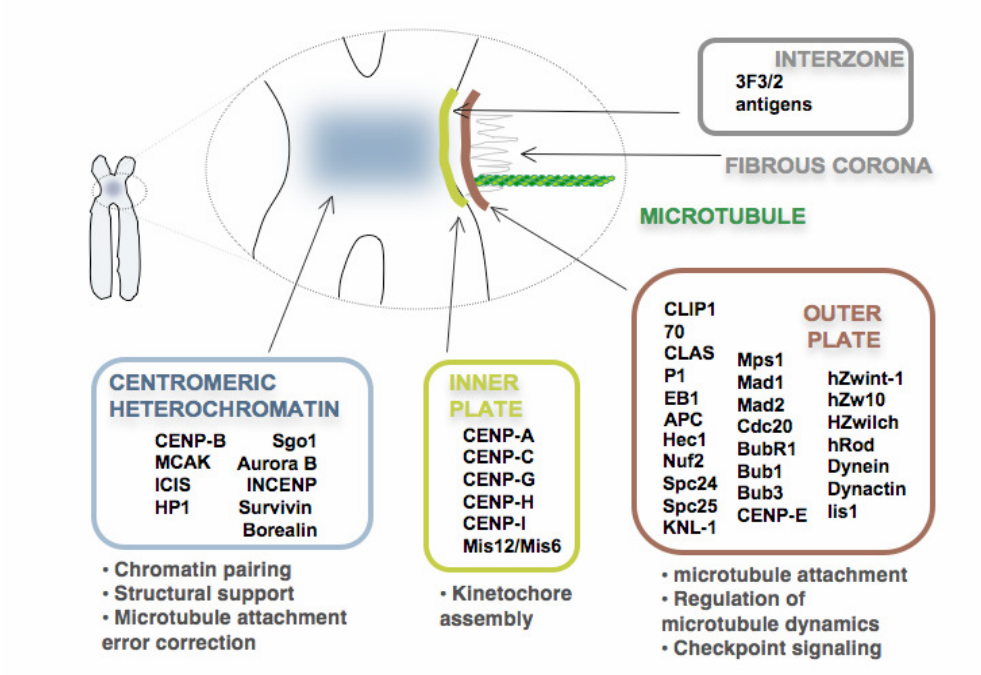


Figure 3. Kinetochore structure (adapted from Maiato et al., 2004).

4.2 CENP-C and the Constitutive Centromere-Associated Network (CCAN)

Some kinetochore components have a distinct temporal order of appearance at the kinetochore. This suggested that the trilaminar kinetochore structure assembles in a step-wise manner. However, a constitutive protein core, recently termed the constitutive centromere-associated network (CCAN) (Cheeseman et al., 2008), remains associated with the centromere throughout the cell cycle. Within this network, CENP-C, CENP-S and CENP-T were identified separately (Moroi et al., 1980; Earnshaw and Rothfield, 1985). CENP-M, CENP-N and CENP-T were found in HeLa cells as part of the CENP-A-nucleosome Associated complex (NAC) (Foltz et al., 2006). Moreover, seven new CENP-A nucleosome distal

components (CAD) were also found associated with the NAC (Figure 4) (Foltz et al., 2006). CCAN components localize to the inner kinetochore and are required for kinetochore integrity (Foltz et al., 2006; Okada et al., 2006), defining the earliest known steps of kinetochore assembly.

In HeLa cells, CENP-A nucleosomes form a complex with CENP-B and CENP-C (Ando et al., 2002) on α-satellite DNA containing CENP-B box consensus sequences (αI-type arrays). Depletion of CENP-A from purified pre-kinetochore chromatin shows that most of the CENP-C, and approximately half of CENP-B, are associated with CENP-A (Ando et al., 2002). In addition to forming a complex with CENP-A and CENP-B, CENP-C also contains a novel DNA-binding domain, which allows a direct interaction with the DNA (Sugimoto et al., 1994; Yang et al, 1996). However, several attempts to identify specific CENP-C-binding sequences have failed.

Although the role of CENP-B in kinetochore assembly is still controversial, studies employing depletion of CENP-C show that this protein is necessary for the formation of a functional kinetochore structure. Injection of anti-CENP-C antibodies into interphase HeLa cells causes mitotic defects starting from prophase (Tomkiel et al., 1994). Kinetochores appear smaller, and microtubule attachments are defective. The cells arrested in metaphase, suggesting that a functional mitotic checkpoint was still able to detect defects in the kinetochore structure. These microinjected cells can eventually complete mitosis with severe chromosome segregation defects. Additional studies support the model that CENP-C is located near the top of the kinetochore assembly pathway. Depletion of CENP-C in human cells results in failure of almost all transient kinetochore proteins, apart from the passenger protein Aurora B (Liu et al., 2006), to localize properly. These proteins included hMis12 (see KMN proteins), the checkpoint proteins BUB1 and BUBR1, which transiently localize to the outer kinetochore while sensing the establishment of correct MT attachments, MCAK (see below) and the motor protein CENP-E. It is possible that CENP-C specifies at least three or four separate sub-branches of the kinetochore assembly pathway.

Comment [??1]: Give brief description

Considering the level of kinetochore disruption following CENP-C depletion, one would expect to see significant structural changes in the kinetochore under these conditions (Liu et al., 2006).

4.3 Other components of the CCAN network

In vertebrates, the CCAN is a multi-complex network that contains at least 14 proteins. As mentioned above, many CCAN components were identified by virtue of being directly or indirectly associated with CENP-A nucleosomes (Figure 4) (Foltz et al., 2006). In another study using human and chicken (DT40) cells, most of the CCAN proteins were found associated with kinetochore CENP-H and CENP-I proteins (Okada et al., 2006). Knock-out studies in chicken DT40 cells allowed the CENP-H/I-associated proteins to be divided into three sub-complexes, based on phenotypic effects and interdependent localization (Okada et al., 2006). These sub-groups are: the CENP-H/I complex, which also contains CENP-K; the CENP-O complex, including CENP-P, -Q, -R and –50; and CENP-M.

Chicken CENP-I was identified and isolated by sequence homology with the fission yeast centromere-associated protein Mis6 (Saitoh et al., 1997). CENP-I and CENP-H localize to the inner kinetochore plate where their localization is mutually inter-dependent (Liu et al.). The CENP-H/I complex is necessary for CENP-C localization in chicken (Nishihashi et al., 2002), but only in interphase, where it appears to be recruited upstream of CENP-C. Depletion of the CENP-H causes arrest prior to anaphase with unaligned chromosomes that fail to attach to the mitotic spindle. After a delay, these cells eventually exit mitosis but with severe chromosome segregation defects (Fukagawa et al., 2001). Similar effects were observed after depleting CENP-I and CENP-K, confirming the interdependent functions of these proteins (Liu et al., 2003; Okada et al., 2006).

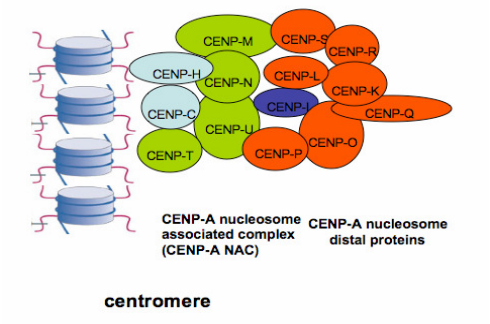


Figure 4. The CCAN. (Adapted from Foltz et al., 2006)

4.4 Kinetochore-Microtubule Network (KMN)

Transient kinetochore proteins start to associate with the CCAN in late G2-phase. The binding of these proteins is essential for assembly the interface between the kinetochore and the mitotic microtubules (KMN) (Figure 5) (reviewed in Fukagawa, 2008). The first complex that binds to the pre-kinetochore structure is the Mis12 complex. The human homologue hMis12 was first identified through a bioinformatics approach (Goshima et al., 2003). So far, 10 proteins have been identified that interact with hMis12: the human Ndc80 complex (hNdc80/HEC1, hNuf2, hSpc24 and hSpc25; (Bharadwaj et al., 2004; McCleland et al., 2004), the predicted human homologue of worm KNL1 (AF15q14); and Zwint and the three previously known components of the Mis12 complex (Cheeseman et al., 2004; Kline et al., 2006).

Although hMis12 was initially reported to localize constitutively at the kinetochore throughout the cell cycle (Goshima et al., 2003; Kline et al., 2006), a later study showed that it is only associated with the kinetochore from late G2 to telophase (Liu et al., 2006). Depletion of any one of the components of the hMis12 complex causes mitotic delay with a significant accumulation of unaligned/mono-oriented chromosomes. After several attempts to congress their chromosomes at the metaphase plate, cells depleted of these proteins eventually exit mitosis with a high number of lagging chromosomes. In contrast to earlier results on Mis12 depletion, which showed no effect on CENP-A levels (Goshima et al., 2003), Kline et al., (2006) found that depletion of hDsn1 leads to decreased levels of kinetochore-associated CENP-A and CENP-H. Kline et al also showed that

levels of the motor protein CENP-E, the kinetochore component Ndc80 (see below) and the checkpoint protein BubRI were also reduced at the kinetochore following Mis12 depletion. A genetic analysis in fission yeast has also showed that Mis12 recruitment does not depend on CENP-A loading, suggesting that CENP-A and Mis12 recruitment pathways are uncoupled (Takahashi et al., 2000). Exactly how the Mis12 complex correlates functionally with the pre-kinetochore structure remains to be elucidated.

In C. elegans, an RNAi-based functional genomic screen allowed the discovery of KNL1 (Kinetochore Null) and five more proteins required for mitotic chromosome segregation (Gonczy et al., 2000; Cheeseman, 2008). The human homologue hKNL1 localizes to the kinetochores in late G2, similar to the subunits of human Mis12 complex, and before Ndc80/HEC1 (Cheeseman et al., 2008). The depletion of hKNL1 in HeLa cells has no effect on the association of CENP-A, CENP-C or the two CENP-H- and CENP-Q-complexes (Cheeseman et al., 2008). However, hKNL1 and hMis12 complexes are dependent on one another for localization at the kinetochore (Cheeseman et al., 2008).

Depletion experiments with hKNL1, CENP-K and Ndc80 have established that hKNL1 and the CENP-H/I complex assist cooperatively the recruitment of the Ndc80 complex to the outer kinetochore (Figure 6) (Cheeseman et al., 2008). In support of this model, the Ndc80 complex physically associates with hKNL1 and hMis12 in the KMN network (Cheeseman et al., 2004; Obuse et al., 2004), and also with CENP-H (Mikami et al., 2005; Okada et al., 2006).

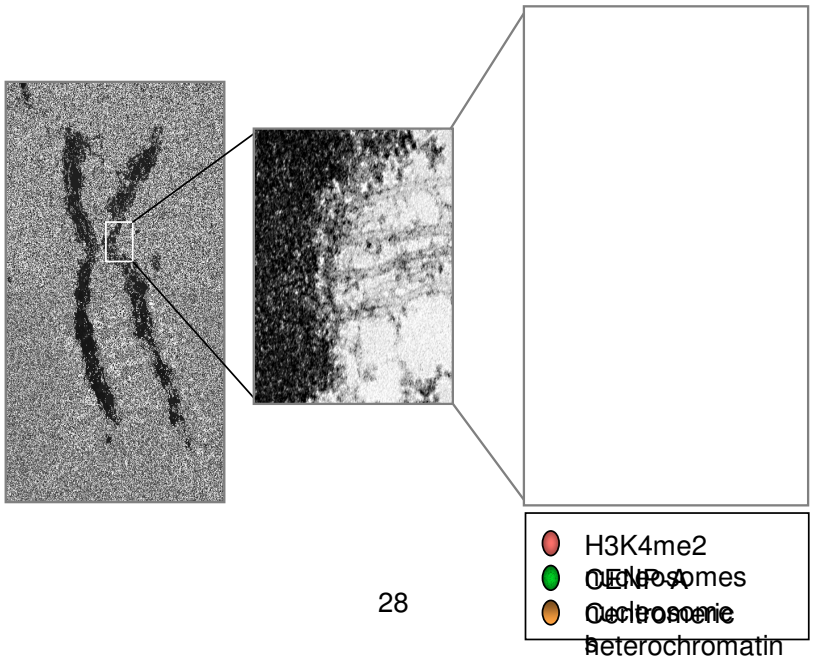


Figure 5. The KMN and microtubule attachment to the outer kinetochore (adapted from Ruchaud et al., 2007).

Microtubules interact at the outer kinetochore with a network of proteins, the KMN network, that are responsible for the highly regulated and dynamical kinetochore-microtubule attachments.

4.5 Ndc80 complex

Several studies of the Ndc80 complex, which contains Ndc80, Nuf2, Spc24 and Spc25, have revealed that this complex is important for the integrity of kinetochore-microtubule attachments and tension (DeLuca et al., 2005). Human Ndc80 complex (Ndc80 is also known as HEC1 for "Highly Expressed in Cancer") is recruited to the constitutive core of the kinetochore in late G2 and, in contrast to other motor and checkpoint proteins, remains stably associated with the outer kinetochore throughout mitosis. The four subunits of the complex assemble into a heterotetrameric rod of about 570 Å in length with a globular head at each end (Wei et al., 2005). Ndc80p and Nuf2p contribute to one head, and Spc24p and Spc25p to the other. The length of the complex is such that it could, in principle, bridge between the outer and the inner layers of the trilaminar kinetochore structure (Wei et al., 2005; Gillett et al., 2004; Ciferri et al., 2005). When microtubules are attached, the complex is likely to be oriented so that the Ndc80/Nuf2 head interacts with the microtubules and the Spc24/25 head is directed towards the centromere (Gillett et al., 2004). Microtubule cosedimentation assays have shown that the Ndc80/HEC1 complex binds directly to microtubules *in vitro* through the Ndc80/Nuf2 heads (Cheeseman et al., 2006), while the Spc24p/25p globular domain does not bind directly to microtubules. Furthermore, the binding of Ndc80/Nuf2 seems to be stabilized by the Mis12/KNL1 complex of the KMN network. Based on *in vitro* co-

sedimentation data, it has been proposed that the KMN network is involved in an array of multivalent, low-affinity interactions that are critical for the association of the dynamic microtubule plus-ends with kinetochores (Cheeseman et al., 2006) (Figure 6).

Figure 6. Model of a kinetochore-microtubule interaction (adapted from Ruchaud et al., 2007).

KNL1 and the Ndc80/HEC1 complex interact with the (+)-end of microtubules and the Mis12 complex at the inner kinetochore. Aurora B kinase, which is part of the Chromosomal Passenger Complex (CPC), regulates the affinity-binding of Ndc80/HEC to the MTs by phosphorylating the NH2-terminus.

4.6 Spindle assembly and dynamics

The mitotic spindle is essential for mitotic cell division, because it is required for the equal distribution of replicated chromosomes to the two daughter cells. The mitotic spindle consists of bundles of microtubules (MT) (dynamic fibres that polymerize from tubulin subunits) and many other proteins that contribute to its function and regulation. Central to the mechanism of mitosis is the polar structure of microtubules. Each microtubule is oriented with its minus-end close to one of the two spindle poles and its plus-end extending away. In addition, the two kinetochores on sister chromatids interact with microtubules originating from opposite poles. These kinetochore-tethered microtubules (kMT) direct the translocation of the

two sister chromatids towards opposite poles during anaphase of mitosis (Rieder and Salmon, 1998).

Located at the centre of the spindle pole in animal somatic cells is the centrosome, an organelle that functions as the major microtubule-organizing center of the cell (Brinkley, 2001; Nigg et al., 2002). One popular model for centrosome-driven spindle morphogenesis is called “search-and-capture” (Kirschner and Mitchison, 1986). This hypothesis pictures the centrosome as generating astral microtubules that undergo cycles of growth and shrinkage, randomly probing the cytoplasm until they encounter a kinetochore and form stable attachments to it. This model for the spindle-organizing function of centrosomes offered a simple explanation for the formation of multi-polar spindles in cells with supernumerary (i.e.more than two) centrosomes. In support of this model, the direct capture of kinetochores by microtubule plus-ends has been observed in newt lung cells (Rieder and Alexander, 1990).

Despite clear evidence for the “search-and-capture” mechanism, it has been calculated that hours should be required to capture all 92 kinetochores in a human cell. Instead, it only takes between 15 and 20 minutes for a cell to saturate all kinetochores with 20-40 kMTs. Furthermore, in acentrosomal cells that lack centrosomes, e.g. like certain plant cells and Xenopus oocytes, a spindle “self-assembles” from MTs that apparently nucleate around the chromosomes (Reviewed in Heald et al., 1997). It therefore appears that two pathways could drive spindle assembly: one dependent on centrosomes and the other independent of centrosomes.

To determine whether the self-assembly pathway of the chromosome-nucleated microtubules also exists in cells that normally contain centrosomes, researchers have developed technologies for removing centrosomes from somatic animal cells entering mitosis. These experiments have shown that, in the absence of centrosomes and astral microtubules, microtubules self-assemble and form bipolar spindles with relatively normal kinetics in fly (Debec et al., 1995) and mammalian cells (Khodjakov et al., 2000; Hinchcliffe and Sluder, 2001). Microtubules bundles can nucleate near kinetochores and elongate by incorporation of new tubulin at their plus-ends

until they encounter astral microtubules emanating from the centrosomes (Maiato et al., 2004). At this point, the newly-formed fibre is captured and transported towards the centrosome by a mechanism that requires the motor protein dynein (Khodjakov et al., 2003); (Maiato et al., 2004).

In summary, the current model for bipolar spindle formation includes microtubules emanating from both centrosomes and kinetochores, which cooperate in order to form a common polarized spindle where each kinetochore is attached to a bundle of microtubules.

4.7 Fine tuning of kinetochore attachments and chromosome bi-orientation

A complex network of kinetochore proteins is responsible for the highly dynamic attachment of the kinetochores to spindle microtubules. Moreover, the regulation of these attachments is essential for the proper behaviour of chromosomes in mitosis.

In mitosis, in a process called chromosome congression, chromosomes gather in the region between the two spindle poles to form the “metaphase plate”. During this process, chromosomes exhibit directional instability – they oscillate between force-generating poleward translocation and antipoleward movement (Skibbens et al., 1993; Khodjakov and Rieder, 1996). These directional switches must be rapid and the sister kinetochores must be coordinated in order to avoid the establishment of incorrect kinetochore-spindle attachments. Nevertheless, erroneous attachments can occur even in normal mitosis (Dong et al., 2007) and must be corrected. Erroneous kinetochore-microtubule attachments can be either syntelic (both sister kinetochores attached to the same pole) or merotelic (one kinetochore attached to both poles). A mechanisms called the “mitotic check-point” has evolved to detect mis-positioned or incorrectly-attached chromosomes and delay the onset of anaphase (mitotic checkpoint) so that these errors can be corrected (Rieder and Salmon, 1998).

For this purpose, the interaction of spindle microtubules with the kinetochore (kMT) is highly regulated (Rieder and Salmon, 1998). The low affinity and cooperativity of the binding of reconstituted Ndc80 complexes to the microtubule lattice offers an efficient way to dynamically regulate this interaction (reviewed in Tanaka and Desai, 2008).

Aurora B is a key factor in proper chromosome biorientation, and its inhibition increases the frequencies of syntelic and merotelic attachments, leading to an overall increase in the number of lagging chromosomes in anaphase (Cimini et al., 2006). Among other functions, Aurora B Kinase regulates the Ndc80/HEC1 complex (Figure 6) (Cheeseman et al., 2002). Disruption of Aurora B-mediated phosphorylation of Ndc80 results in mitotic delay, defects in chromosome alignment at the metaphase plate and an increased frequency of lagging chromosomes in anaphase (DeLuca et al., 2006). One proposed explanation for these phenotypic effects is that Aurora-B phosphorylation regulates the interaction of the Ndc80 complex with the kMTs. In support of this model, inhibition of Aurora B kinase stabilizes kMT turnover in mitosis and induces errors in chromosome segregation (Maiato et al., 2004; Cimini et al., 2006).

Another important player in the highly regulated kinetochore-microtubule interaction is MCAK (mitotic centromere-associated kinesin). MCAK is a potent depolymerase of microtubules (Desai et al., 1999; Hunter et al., 2003) and it has been observed to track with polymerizing MT tips (Moore et al., 2005) in a manner that is negatively regulated by phosphorylation of its NH-terminal domain. The activity of MCAK is inhibited by Aurora B *in vitro*, and it was shown that this inhibitory phosphorylation occurs during the process of bipolar attachment and chromosome congression (Lan et al., 2004). Aurora B and MCAK are both enriched at metaphase-aligned kinetochores with merotelic attachments. Furthermore, both MCAK localization and attachment resolution depend on Aurora B phosphorylation (Figure 7) (Knowlton et al., 2006). MCAK delocalization from the kinetochore by a dominant negative mutant causes an increase in the number of syntelic and merotelic

attachments, which leads to an increase in the number of unaligned and lagging chromosomes (Kline-Smith et al., 2004).

It is not clear how inhibition of the depolymerizing activity of MCAK by Aurora B helps to resolve merotelic attachments. However, Aurora B phosphorylation may mediate the localization of MCAK to specific sub-centromeric regions where its function is required (Figure 7) (Andrews et al., 2004; Knowlton et al., 2006).

In conclusion, Aurora B seems to control chromosome biorientation by phosphorylating a number of substrates and in turn regulating plus-end MT dynamics and kinetochore-spindle attachments.

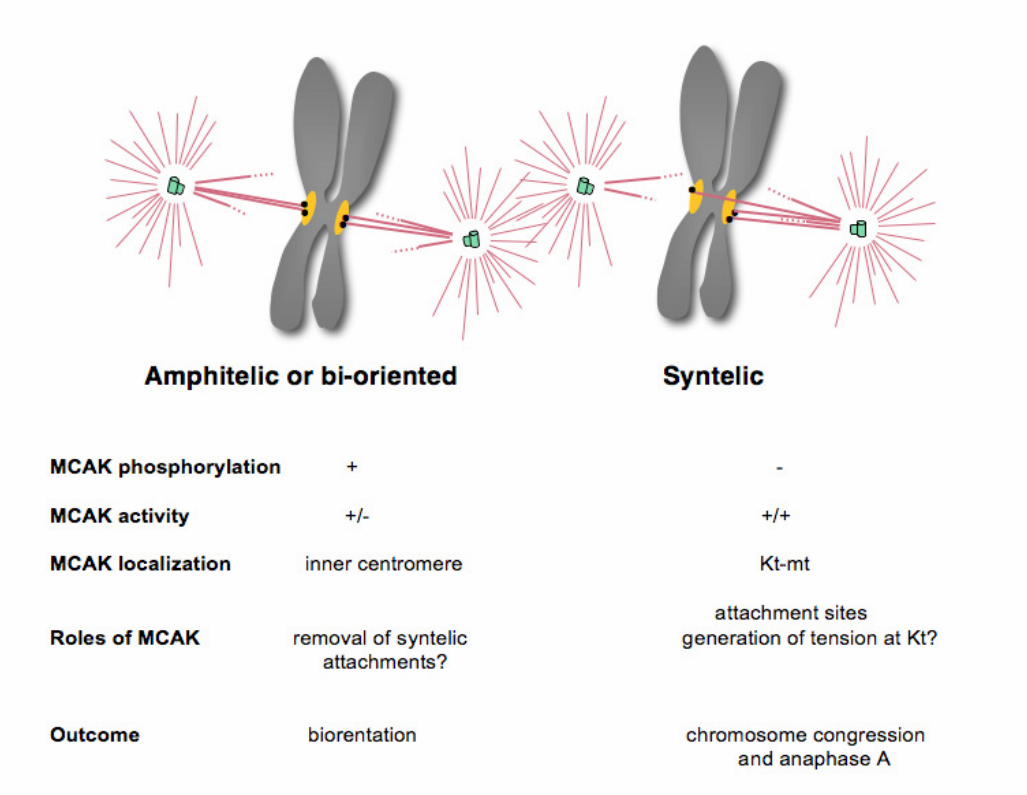


Figure 7. Role of MCAK in the establishment of correct kinetochore-microtubule attachments.

Phosphorylation and/or localization of MCAK at the centromere regulate the formation of bipolar microtubule attachments.

5. A higher level of genetic regulation: chromatin

5.1 Different types of chromatin

In eukaryotic cells, genomic DNA is folded by histone and non-histone proteins to form chromatin. Despite the important amount of coding information present in the DNA sequence, multiple levels of information and regulation of genic expression are associated with chromatin organization. This is referred to as epigenetic regulation. Histone modification, DNA methylation and chromatin-remodelling mechanisms are all part of the epigenetic regulation of discrete chromatin domains (reviewed in Jenuwein and Allis, 2001).

The distinction between heterochromatin and euchromatin was first proposed on the basis of different compaction of DNA in interphase (Heitz E., 1928). Today, 60 years after this first observation, it is widely recognized that euchromatin is in general less condensed, more accessible to enzymes and more transcriptionally active. By contrast, heterochromatin is more condensed, less accessible and contains more structured nucleosomal arrays (reviewed in Huisinga et al., 2006). Chromosomal regions that are characterized by a high density of repetitive sequences – for example telomeres - are usually associated with heterochromatin (Martens et al., 2005; reviewed in Grewal, 2007). These regions of heterochromatin remain condensed throughout the cell cycle and are referred to as “constitutive” heterochromatin. However, another type of heterochromatin, termed “facultative”, can be nucleated at specific loci and is regulated by cellular signals or gene activity (reviewed in Trojer and Reinberg, 2007).

The presence of heterochromatin at chromosomal loci was originally discovered in Drosophila by a phenotypic assay for mosaic gene silencing called position-effect variegation (PEV) (Muller HJ, 1932; Schultz, 1936). The association of PEV with heterochromatin led scientists to hypothesize that an important characteristic of heterochromatin is its ability to spread over neighbouring sequences and repress (silence) transcription in a sequence-independent manner (Demerec, 1940; Hartmann-Goldstein, 1967).

Multiple pathways of histone modification and DNA methylation in higher eukaryotes contribute to heterochromatin assembly (Maison and Almouzni, 2004). The initial targeting of heterochromatin to nucleation sites is distinct from its spreading and maintenance. Following nucleation, heterochromatin spreading is characterized by a self-sustained processive behaviour. Methylation of Lysine 9 on Histone H3 (H3K9me), a histone modification enriched on heterochromatin, is catalyzed by the histone methyltransferase SuVar(3-9) and acts as a target for the binding of the chromodomain of Heterochromatin Protein I (HPI). HPI has a chromoshadow multimerization domain and the ability to bind other proteins involved in heterochromatin formation. Thus, HPI acts as an assembly platform for the stabilization and spreading of heterochromatin (Smothers and Henikoff, 2000; Lechner et al., 2005).

5.2 The centromeric and pericentromeric chromatin

The centromere region is broadly organized, with respect to its chromatin structure, into two major domains: 1. a central domain (CEN) that is defined by its role in kinetochore assembly; and 2. a heterochromatin block (pericentromeric heterochromatin) that flanks one or both sides of the CEN domain (reviewed in Choo, 2001). The role of pericentromeric heterochromatin for centromere function has been best studied in fission yeast. In *S. pombe*, recruitment of the HPI homologue Swi6 to pericentromeric heterochromatin is required for later recruitment of the cohesin subunit Rad21 and the maintenance of sister chromatid cohesion (Bernard et al., 2001; Nonaka et al., 2002). In fact, cells lacking *swi6* are defective in centromere cohesion between sister chromatids, but arm cohesion is conserved (Bernard et al., 2001; Nonaka et al., 2002) Furthermore, yeast *rad21 swi6* double mutants are synthetically lethal, as they lose both centromeric and arm cohesion (Bernard et al., 2001).

Using ChIP analysis to map the location of cohesin subunits on the chromosomes, several laboratories found that centromeres are the

preferential site of cohesin assembly. Furthermore, the association of cohesin with the centromere is dependent on the presence of a functional kinetochore (reviewed in Sullivan, 2001).

CEN chromatin is organized into distinctive sub-domains of CENP-A-containing nucleosomes interspersed with differently modified H3 nucleosomes (Figure 8). Analysis of extended chromatin fibres in human and fly cells suggests that nucleosomes containing histone H3 dimethylated on Lysine 4 (H3K4me2) are interspersed with the CENP-A containing nucleosomes (Sullivan and Karpen, 2004; Cam et al., 2005; Lam et al., 2006). Recent studies also indicate that clusters of H3K9me3 chromatin are present with CENP-A-containing chromatin at centromeric satellite DNAs (Nakashima et al., 2005; Lam et al., 2006). Also, HPIα and HPIγ were recently found to co-purify with centromere component hMis12 (Obuse et al., 2004). These studies suggest that sub-regions of heterochromatin might be directly linked to centromeric function. By contrast, other reports suggest that the H3K9me3 chromatin antagonizes the formation of CENP-A chromatin on alphoid DNA (Nakashima et al., 2005; Okamoto et al., 2007). Although the role of H3K9me3 chromatin at the centromere remains to be clarified, it has recently been suggested that a balance between this chromatin and CENP-A-chromatin is maintained, possibly by the activity of CENP-B (Okada et al., 2007). An epigenetic regulation of the different nucleosome clusters might be the key mechanism that preserves centromere identity.

Regardless of the distribution of the different types of nucleosomes in the CEN chromatin, it seems that the vertebrate centromere folds into spatially distinct domains (Blower et al., 2002; reviewed in Vagnarelli, 2008). The internal domain (inner centromere) is enriched in H3-nucleosomes and interacts with HPI/cohesin complexes, possibly regulating the function of the chromosomal passenger complex and cohesion between sister chromatids (Vagnarelli and Earnshaw, 2001). The outer centromeric domain contains

CENP-A and functions as a substrate for the assembly of a kinetochore structure (Figure 8).

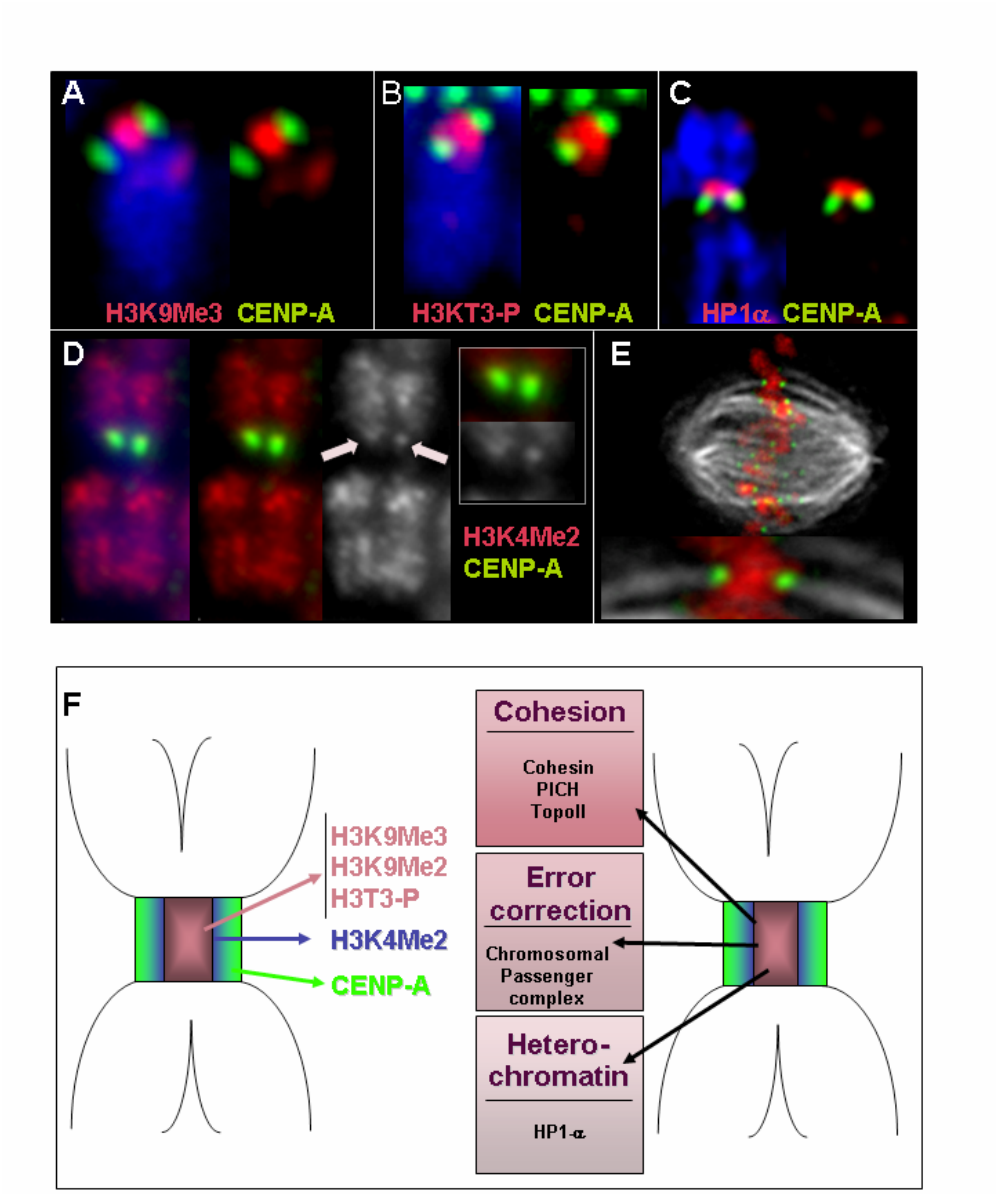


Figure 8. Organization of chromatin at the centromere.

Heterochromatin-type H3-nucleosomes and HPIα-associated chromatin localize internally to CENP-A-nucleosomes (A-C). K4me2-modified H3-nucleosomes localize more closely to CENP-A (D). (F) Model of the distribution and possible roles of the different types of chromatin within the centromere (Adapted from Vagnarelli et al., 2008).

5.3 Nucleation of heterochromatin

The RNAi machinery and DNA-binding proteins work in distinct pathways to direct nucleation of heterochromatin (reviewed in Grewal and Jia, 2007). The formation of constitutive heterochromatin seems to be unequivocally linked to the presence of arrays of repetitive sequences that nucleate its formation (Dorer and Henikoff, 1994; Selker, 2002). In plants, Drosophila and fission yeast, this mechanism relies upon the generation of small interfering RNAs (siRNAs) that match the target genome sequences (Figure 9) (Reinhart and Bartel, 2002; Aravin et al., 2003). It has been proposed that the RNAi machinery mediates the direct targeting of heterochromatin nucleation at specific loci in S. pombe and other organisms (reviewed in Grewal and Jia, 2007).

On the other hand, DNA-binding proteins may promote heterochromatin nucleation in a specific region by directly recruiting HPI and SUV39 proteins (Nielsen et al., 2001; Schultz et al., 2002). In S. pombe, the presence of constitutive heterochromatin across a 20 kb domain in the mating-type region (mat2/3) is critical for transcriptional silencing and suppression of recombination (Grewal and Klar, 1997). The binding of transcription factors to specific DNA sequences cooperates with Clr3 (a histone deacetylase enzyme) to recruit Clr4 (a H3K9-specific methyltransferase) and stabilize H3K9 methylation, thereby nucleating and maintaining heterochromatin (Figure 9) (Jia et al., 2004; Kim et al., 2004; Yamada et al., 2005).

DNA-binding proteins and local cis-acting sequences can also promote the formation of facultative heterochromatin that regulates gene expression. E2F1 is a component of the E2F family of transcriptional regulators, which

stimulate transcription of several genes in the apoptosis pathway (Nahle et al., 2002). One of the principle regulators of E2F1 is the retinoblastoma protein pRB (Nevins, 1998) which binds to E2F1 and recruits HDAC1 and SUV39H1 to the target promoters, thereby changing the chromatin structure and repressing transcription (Figure 9) (reviewed in Dimova and Dyson, 2005). In addition, it was recently suggested that the nuclear corepressor KAP1 (KRAB-asssociated protein 1, also known as TIF1β) negatively regulates E2F1 by changing chromatin structure (Wang et al., 2007).

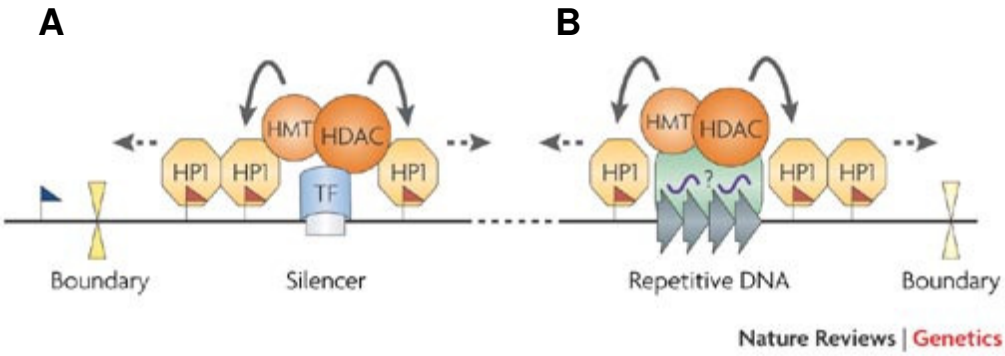


Figure 9. Nucleation and spreading of heterochromatin.
Histone-modifying enzymes are targeted to specific loci by transcriptional factors **(A)** or by siRNAs generated at repeated DNA sequences **(B)** to nucleate heterochromatin. The following binding of HP1 to methylated histone tails ensures the spreading and maintenance of the heterochromatin. (from Grewal and Jia *Nature Reviews Genetics* **8**, 35–46, January 2007)

5.4 KAP1-mediated gene silencing

KAP1 provides an interesting example of chromatin-mediated gene silencing. KAP1 was initially identified as a corepressor of the large class of KRAB-containing transcriptional repressors (Friedman et al., 1996). KRAB (Kruppel-associated box) is a potent repression domain identified in one-third of all transcriptional repressors of the zinc-finger class (Bellefroid et al., 1991). KAP1 localizes in the nucleus as a homo-oligomeric complex of 3 or 6

polypeptides (Peng et al., 2000), and contains an interesting combination of protein domains (Figure 10). Starting from the NH2-terminus, KAP1 presents a RING finger, B-boxes and a leucine zipper coiled-coil region (RBCC domain). This region is both necessary and sufficient for the interaction with the KRAB domain and for oligomerization (Peng et al., 2000). Interestingly, following the RBCC region there is an HPI-binding consensus sequence which directly binds to HPI (Lechner et al., 2000). At the C-terminus, KAP1 contains a PHD (plant homodomain) finger and a bromodomain, which cooperatively function as a transcriptional repression unit by recruiting the histone deacetylase complex NuRD and the H3K9-specific methyltransferase SETDB1 (Schultz et al., 2001; Schultz et al., 2002). Two regions of KAP1 are involved in chromatin-mediated transcriptional silencing: the HPI-binding region and the PHD/bromodomain. It has been proposed that the binding to DNA-associated transcriptional repressors targets KAP1 to specific chromosomal loci. The two chromatin-modifying units of the protein then alter the chromatin structure, inhibiting transcription. In fact, targeting of KAP1 to reporter genes resulted in both transient and stable epigenetic silencing (Sripathy et al., 2006; Ayyanathan et al., 2003). Thus far, apart from regulating E2F1, KAP1 has been found having a role in the regulation of the DNA damage response, where it interacts with p53 and inhibits its activity (Wang et al., 2005).

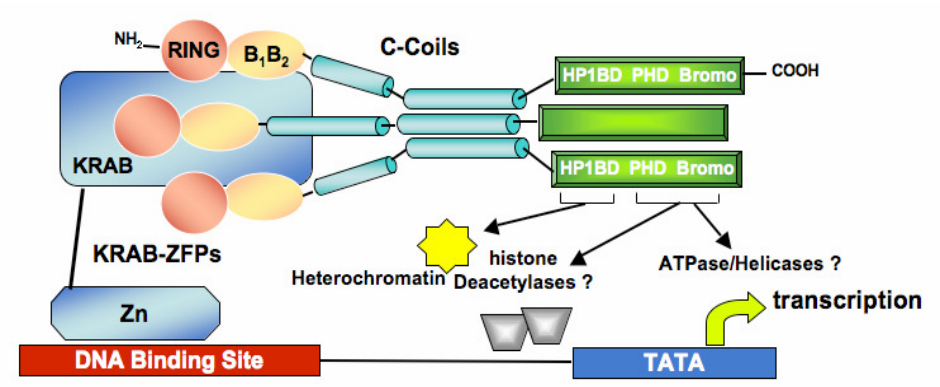


Figure 10. Domain composition of KAP1 (adapted from Peng et al., 2000)

6. Aims

My PhD work was focused on the study of a second generation of Human Artificial Chromosome (HAC), containing sequences that allow its manipulation *in vivo.* This study was part of a scientific collaboration between Prof. WC Earnshaw and Dr. V Larionov's laboratory at the National Institute for Health (NIH) in Bethesda-MA (USA). I will describe and discuss the detailed characterization of a HAC that was obtained in human fibrosarcoma HT1080 cells by Dr. Megumi Nakano, at the NIH. In Chapter III I will analyze the construction, stability and manipulation of the HAC obtained by Dr Nakano. In particular, I will show that the DNA of the newly constructed HAC is accessible to the binding of protein constructs which bind specifically to the HAC. We also succeeded in manipulating the chromatin associated with the HAC by targeting chromatin modifiers like KAP1, HPI and Ezh2. In the second results chapter, I will describe the inactivation of the synthetic kinetochore assembled on the HAC. For this analysis, I transferred the synthetic HAC into a HeLa-based human cell line, the first time a HAC is described in a human cell line other than HT1080. The disruption of the HAC's kinetochore, mediated by protein targeting to its alphoid repeats, allowed the first direct dissection, *in vivo,* of the tight link between the assembled kinetochore complexes and the underlying centromeric chromatin.

Part of the work described in the first results chapter was done in collaboration with Dr. M Nakano. This joint work was published in the issue 2008 Apr;14(4):507-22 of Developmental Cell.

II. Materials and Methods

1. **Construction of tetO dimer alphoid BACs**

*****The alphoid^{tetO} monomer sequence was assembled by co-ligation of 5 double-stranded oligomers and cloned into pBluescript using the *XhoI* / *SalI* sites. A naturally occurring alphoid monomer (168bp) containing a consensus CENP-B box was isolated from p17H8 (Waye and Willard, 1986). The 2.7 kb higher-order repeat was digested with *BsmI* and the appropriate DNA fragment was isolated after agarose gel electrophoresis. The fragment was blunt-ended with T4 polymerase and cloned into the *SmaI* site of pBluescript. After amplification of the monomer by PCR to introduce *SalI* and *XhoI* sites, it was inserted into pBluescript next to the tetO monomer.

Subsequent cycles of digestion and cloning the *SalI-XhoI* site of pBluescript yielded p3.5a, containing 10 alphoid^{tetO} dimer fragments. The extension of the tetO dimer alphoid repeat was carried out by rolling-circle amplification (RCA) for the circularized template of the alphoid^{tetO} 10-mer excised from p3.5a, using f29 DNA polymerase. Resulting RCA products were cloned into a targeting vector in yeast by transformation-associated homologous recombination (TAR) according to previously described methods (Ebersole et al., 2005). The targeting vector (RSA/SAT43) contains YAC (*HIS3*, *CEN6*, *ARSH4*) and BAC (*Cm*, *ori F*) cassettes as well as a mammalian selectable marker (*bsr*).

Purified genomic DNA from the yeast clones was electroporated into *Escherichia coli* cells (DH10B, Invitrogen). The insert size of the synthetic alphoid^{tetO} DNA array was analyzed by PFGE (CHEF, Bio-Rad) after *NotI* digestion of BAC DNA.

* Quoted from the paper in Appendix.

2. BAC transfection.

The alphoid^{tetO} BAC DNAs were purified using a Qiagen large construction kit (QIAGEN). Using 4.5 µl of Lipofectamine Reagent (Invitrogen Corporation), 0.4 µg of purified BAC DNA was transfected into HT1080 cells. Bs-resistant cell lines were selected with 4 µg/ml blasticidin S hydrochloride (MP

Biomedicals, Inc.) and analyzed by FISH. To obtain homogeneous populations of sub-lines containing HACs, single colonies were picked up from the original alphoid^{tetO} HAC clones AB 2.2.18 and AB 2.5.4. Throughout the sub-cloning process, cells were cultured in non-selective medium.

3. ***De novo*** **HAC formation analyses by fluorescence in situ hybridization**
(FISH).

Standard FISH techniques were carried out for the alphoid^{tetO} BAC transformed cell lines as previously described (Masumoto et al., 1989). The probes used were PCR products of p3.5 for the alphoid^{tetO} dimer and RSA/SAT43 for the BAC vector DNA. Alphoid^{tetO} dimer template was amplified by PCR using TaKaRa LA Taq (Takara Bio Inc.) with M13 universal and reverse primers. Alphoid DNA hooks were eliminated from the BAC vector by restriction enzyme treatments and the fragment containing the YAC and BAC cassettes was purified from the gel. PCR amplified alphoid^{tetO} and the BAC vector DNA fragments were labeled using a nick-translation kit with digoxigenin-11dUTP or biotin16-dUTP (Roche Diagnostics). Images were captured using a cooled-charge-coupled device (CCD) camera (Cool SNAPHQ, Photometrics) and analyzed by IPLab software (Signal Analytics). Loss rates (R) of HACs per generation were calculated using the following formula:

$$N37 = N0 \times (1-R)37.\ N37$$

is the average number of HACs per cell from 20 observed metaphase cells at day 37 and N0=1 in this case because of the sub-cloning.

4. Cell lines. HT1080 cells (ATCC CCL121) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Corporation) supplemented with 10% (v/v) tet system-approved fetal bovine serum (Clontech Laboratories, Inc.) at 37℃ in 5% CO_2. To construct cell lines expressing tetR-fusion proteins, mRFP-fused tetR protein expression vector, pRFP-tetR

or tetRfused VP16 transcriptional activation domain expression vector, ptTA was introduced into HT1080 sub-lines containing the alphoid^{tetO} HAC using the Polyfect Transfection Reagent (QIAGEN) according to the manufacturer's instructions. After transfection, cells were exposed to 4 µg/ml of puromycin for 2 days. A retroviral vector system was also used to obtain cell lines expressing tetR-fusion proteins. Infectious virus particles incorporating vectors expressing tetR-fusion proteins were generated according to the manufacturer's instructions and used to infect the AB2.2.18.21 or AB2.5.30 cell line. After infection, cells were treated with 150 µg/ml or 400 µg/ml of geneticin, respectively. The control HAC cell line W0210R-8 containing one copy of a stable HAC derived from synthetic wild-type 11-mer alphoid (alphoid^{11mer}) BAC was described previously (Ohzeki et al., 2002).

To obtain HeLa-HT1080 heterokaryon cell-lines, HeLa cells resistant to the drug Geneticin were plated with AB2.2.18.21 cells in a ratio 50%:50% in a 10cm Petri dish and let to adhere. Next day, cells were washed with 1xPBS (Invitrogen) and incubated with a 50% solution of PolyethylenGlycol (PEG) in PBS for 2' at room-temperature, and then extensively washed with 1x PBS before adding fresh medium. HeLa-HT1080 hybrid clones were selected with 500 µg/ml of Geneticin and 8 µg/ml of Blasticidin (for selecting clones carrying the alphoid^{tetO} HAC). The 1C7 cell-line was selected and cultured in RPMI medium (Invitrogen Corporation) supplemented with 10% of fetal bovine serum (Invitrogen) and with 500 µg/ml of Geneticin and 8 µg/ml of Blasticidin.

5. Construction of tetR-fusion protein expression vectors.

For targeting constructs into a retroviral vector, the tetR gene was amplified from pTetON/OFF vectors (Clontech) with primers reTetR-R and reTetR-F, and inserted into the retroviral vector pFB-Neo (Stratagene) using *EcoRI* and *BamHI* (pFB-tetR-Neo). The VP16 coding sequence was amplified from pTet-ON (Clontech), with PCR primers VP16-S (see table) and VP16-N and inserted into the pFB-tetR-Neo using *StuI* and *NotI* (pFB-tTA-Neo). The tTS coding sequence was amplified by PCR from pTet-tTS with primers, re-tetR-

F and retTS-R and inserted into the pFB-Neo using *EcoRI* and *XhoI* (pFB-tTS-Neo). The EYFP coding sequence was amplified by PCR from pEYFP-C1 (Clontech) and inserted into the pFB-tetR-Neo and pFB-tTS-Neo using *StuI* and *XhoI* (pFB-tetR-EYFP-Neo and pFB-tTS-EYFP-Neo), or inserted into the pFB-tTANeo using NotI (pFB-tTA-EYFP-Neo). tTSmut has two amino acid mutation in KRAB-AB domain consensus sequence in SDkid-1 of tTS (Agata et al., 1999; Matsuda et al., 2001). The pFB-tTSmut-Neo and pFB-tTSmut-EYFP-Neo were constructed by PCR using Phsion Site-Directed Mutagenesis Kit (NEB) with PCR primers mKRAB-1 and mKRAB-2, and templates pFB-tTS-Neo and pFB-tTS-EYFP-Neo.TetR-fusion protein genes and the neomycin resistance gene were cloned into a retrovirus vector bearing an internal ribosome entry sequence (IRES). Virus-infected cells were maintained in medium containing neomycin and/or 1 µg/ml of doxycycline.

For the targeting constructs into a non retroviral background, the tetR coding sequence of *E. coli* Tn10 was cloned with and without the stop codon into pZeoSV(-) (Invitrogen) using *EcoRI* and *BamHI*. The mRFP-TetR (in pZeo) was obtained by blunt ligating the mRFP gene into the XhoI site of pZeo(-), upstream of the TetR sequence. The EYFP:TetR targeting construct was obtained by cutting the TetR sequence from pTet-tTS (Clontech) with NdeI/SpeI and ligating the fragment into NdeI/NheI sites of pEYFP-C1 (Clontech). To this plasmid, a cassette expressing resistance to Puromycin was cloned in the AflII site of the vector. All other TetR:YFP-fused constructs were cloned either sticky or blunt in the BglII site in the MCS of the vector, downstream of the TetR:YFP sequence. Cells transfected with these vectors were selected with 1.5-3 µg of Puromycin.

OLIGOS USED

5'-GAAAGGCCTCTTAAATGTGAAAGTGGGTCC-3'	VP16-S
5'-GATCGCGGCCGCTCTACCCACCGTACTCGTCAATTC-3	VP16-N
-CGCTCGAGCTAAGGCCTCCAGG GATCCTCTCCTTGCTGC-3'	retTS-R
5'-GCTGCGGCTGTGCTCTTTACTCGGGACGAG-3'	mKRAB-1
5'-TTCAAATGTCACTGACACTGCTAG-3	mKRAB-2

5'-ACGAATTCATGTCTAGATTAGATAAAAGTA-3'	Re-tetR-F
5'- GAGGATCCCTAAGGCCTCTTTCTCTTCTTTTTTGGTTTA – 3'	Re-tetR-R
5'- TTCGGCCGCAGCCTCGGCCT – 3'	KRBD_1_Fwd
5'- GAGGGGCCATGGGTGCAGGG – 3'	KRBD_1_Rev
5'- ATGGCCCCTCCAAGAGCCCC – 3'	HPBD_1_Fwd
5'- CCCTCCGCAAGAGCCATAAGC – 3'	HPBD_1_Rev
5'- GGTGGCCCGGGAACCCTGGA – 3'	PHD_1_Fwd
5'- GGGGCCATCACCAGGGCCAC – 3'	PHD_1_Rev
5'- AGGATCCAAATGGGAAAGAAAACC – 3'	HPIf
5'- TGGTACCTTTAGCTCTTTGCTGT – 3'	HPIr
5' CCACTCCCTATCAGTGATAGAGAA – 3'	Tet-1
5'- TCGACTTCTGTTTAGTTCTGTGCG – 3'	Tet-3
5'- CAACTCCCAGAGTTTCACATTGC - 3'	17alpF
5'- GGAAACTGCTCTTTGAAAAGGAACC – 3'	17alpF
5'- GTGACGATGGAGTTTAACTCAGGG – 3'	X3-F
5'- GCTTTCCGTTCAGTTATGGGAAGG – 3'	X4-F
5'- CAGGAGAAATCATTTCGGCAGTAC – 3'	Bsr-R
5' TCCATTCGAAACTGCACTACCA – 3'	Bsr-F

6. Indirect immunofluorescence.

Cells expressing YFP- or mRFP-fused targeting constructs were cultured on poly-D-Lysine-coated coverslips, fixed in 4% formaldehyde for 10 min, (and/or treated with methanol for 5 min and dried). The coverslips were then treated with 0.5% (or 0.2%) Triton X-100. Antibodies used were anti-CENP-A (mAN1; Valdivia), anti CENP-C (Ra1; polyclonal c 554), anti-GFP monoclonal antibody (Invitrogen), anti CENP-H (polyclonal bleed R1276), anti-HEC1 (9G3.23 GeneTex, Inc S Antonio USA) and/or anti-GFP polyclonal antibody (Medical & Biological Laboratories co., ltd, Japan). Images were captured using a Zeiss microscope (Axiophot) equipped with a cooled-charge-coupled device (CCD) camera (Cool SNAP HQ, Photometrics) and analyzed by IPLab software (Signal Analytics). Also a DeltaVision (Applied Precision, Issequah, WA) microscope equipped with a CoolSNAP HQ camera was used for image acquisition and analysis.

7. ImmunoFISH and cytological preparations.

ImmunoFISH experiments were performed on chromosome spreads. Cells

from alphoid^{tetO} HAC cell line AB2.2.18 were incubated for 4 hours in 0.1 µg/ml colcemid and after mitotic shake-off, mitotic cells were resuspended in 75 mM KCl at 37°C and incubated for 10 min. After centrifugation, mitotic cells were fixed for 15 min with cold (-20 °C) MeOH. Mitotic spreads were transferred by dropping onto a clean glass slide, dried and incubated in PBST (1x PBS + 0.05% Tween 20) for 5 min. After pre-block with 1% BSA in PBST for 30 min at 37 °C, incubation with primary antibody was done o/n at 4 °C and with secondary for 30 min at 37 °C. After the second incubation, cells were fixed again with 4% PFA for 8 min, washed twice with 2x SSC (0.3M NaCl, 0.03M NaCitrated pH 7.0) buffer for 5 min and EtOH dehydrated. Another wash was done with 2x SSC for 45 min at increasing temperature from 25 to 70 °C, followed by EtOH dehydration. DNA was denaturated with 0.1 M NaOH for 10 min, the slides were washed 3 times with 2x SSC and dehydrated. BAC-probe (obtained using Bionick Labelling kit from Invitrogen) was denaturated and applied to the slides, which were incubated for 2 min on a thermoblock at 75 °C. Hybridization was o/n at 39 °C in a humidified chamber. The following day slides were washed 3 times for 5 min with 2x SSC at 45 °C and again for 5 min at room temperature. Incubation with FITC-avidin (Molecular Probes, Inc.) 1:500 for 30 min at 37 °C was followed by incubation with biotinylated anti-avidin antibody (Vector) (1:100) for 30 min at 37°C and by another final incubation with FITC-avidin. Slides were finally washed for 5 min with 2x SSC and mounted with VectaShield (Vector).

ImmunoFISH on extended chromatin fibers was performed as following. Asyncrounously growing cells were washed with 1xPBS, tripsinized and collected at 1200 g for 4 min. Cells were then resuspended in 75 µM KCl warmed at 37°C and incubated for 10' at RT ($5*10^4$ cells/125 µl) and cytospun on glass slides at 2000 r.p.m for 5 min. After leaving the cells to dry for 10 min, slides were treated with ULB buffer (25 mM Tri-HCl pH 7.5, 0.5 M NaCl, 1% Tryton X-100, 0.5 M Urea) for 15 min at RT and fixed with ice-cold 100% methanol for 10 min. Slides were then air-dried and washed for 5 min with 1xPBS + 0.05% Tween-20 (PBST). The pre-block for the

immunostaining was done with 0.1% BSA in 0.1% milk (in PBST) for 30 min at 37°C. Primary antibody imcubation was performed o/n at 4°C and the secondary antibody at 37°C for 40 min (antibodies were resuspended in PBST). After immunostaining, two washes of 10 min with PBST were followed by Carnoy's fixation for 10 min in ice and then slides were dried. Next step was a wash of 30 min at 37°C with 0.1% Tryton in 2x SSC plus another wash of 5 min with 2x SSC at RT. A step of EtOH dehydration was followed by denaturation with 70% formamide (in 2x SSC) at 78°C for 6 min, EtOH dehydration and finally the DNA hybridization probe (denaturated at 95°C for 5 min) was applied onto the glass slide. The hybridization was performed o/n at 39°C. Next day slides were washed two times with 50% formamide (in 2x SSC) for 7 min each at 37°C and two more times with 2x SSC. The rest of the procedure is identical as for ImmunoFISH.

8. The analysis of HAC loss rate (R) in the TetR-fusion protein expressing cells with real-time PCR.

AB2.2.18.21 cells expressing tetR, tTA and tTS fusion proteins were constructed by retroviral vector gene expressing system. Infected cells were treated by geneticin and/or 1 mg/ml of doxycycline for 30 days (and 7 or 14 days for tTS expressing cell). The genomic DNAs of each cell lines were purified using DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Purified genomic DNAs were sonicated (Bioruptor sonicator, Cosmo Bio, Japan) for 3 min. 100 ng of genomic DNA was used for analysis by real-time PCR with primers, tet-1 and tet-3 using the iCyclerIQ^{TM} MultiColor Real Time PCR Systems (Bio-Rad). The following primer sets were used: 5SDNA-F1 and 5SDNA-R1 for 5S ribosomal DNA, tet-1 and tet-3 for the alphoid^{tetO} dimer. The HAC copy-number of 1C7 cells was measured as described before. Although, tetR, tTA and tTS constructs were cloned in a pEYFP-C1 vector (Invitrogen) and cells were transfected with FugeneHD at 90-95% confluency. Transfected cells were selected with 3 μg/ml Puromycin 24 hours after transfection, and cultured for the required time with 1.5 μg/ml

Puromycin. Loss rates (R) of HACs per generation were calculated using the formula described above.

9. The analysis by real-time PCR of the HAC loss rate (R) in subclones expressing the tTA.

Genomic DNAs of mRFP-tetR or tTA expressing subclones were purified and processed as described above. Additional primer sets included, 17alpF (5'-CAACTCCCAGAGTTTCACATTGC-3') and 17alpR (5'-GGAAACTGCT CTTTGAAAAGGAACC-3') for chromosome 17 alphoid DNA, X3-F (5'-GTGACGATGGAGTTTAACTCAGGG-3') and X4-R (5'-GCTTTCCGTTCAGT TATGGGA AGG-3') for chromosome X alphoid DNA.

10. Analysis by FISH of the HAC loss rate (R) in cells expressing TetR-fusion proteins.

AB2.2.18.21 cells were transfected with Lipofectamine2000 (Invitrogen) at 90% confluence and after 24 hrs puromycin was added to the medium at a concentration of 1.5 mg/ml. After 24-30 hrs the medium was changed and the cells incubated for 4 days before being selected again with the same concentration of Puromycin for 24-30 hrs. After the second selection, cells were incubated in fresh medium for 5 days, plated on poly-lysine-coated slides and processed for fluorescence *in situ* hybridization (FISH) using a BAC-based probe. The slides, fixed with Carnoy's fixative, were left to age o/n. The probe was denatured for 5' at 95° and added to the slides, which were incubated at 72°C for 2' before o/n incubation at 39°C. After washes with 0.1 x SSC (20 x SSC: 3 M NaCl, 0.3 M NaCitrate) at 65°C followed by a wash with 4 x SSC + 0.1% Tween20 at RT, slides were incubated (with intervening washes), successively, with FITC-avidin, biotinylated-anti-avidin and FITC-avidin. Slides were mounted with VectaShield. FISH procedure on 1C7 cells was identical as described above. However, 1C7 cells were transfected with FugeneHD at 90% confluency, and selected with 3 μg/ml Puromycin 24 hours and 5 days after transfection.

11 Quantification of transcripts derived from alphoid^{tetO} HAC.

Real-time RTPCR was carried out using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) according to the manufacturer's protocol, using total RNA prepared with the SV Total RNA Isolation system (Promega). Reverse transcription and PCR were done with the following primer sets: hActin-a (5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3') and hActin-b (5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3') for human actin, 11-10R and mCbox-4 for 11-mer of chromosome 21 alphoid DNA (alphoidchr. 21), tet-1 and tet-3 for the alphoid^{tetO}; bsr-F and bsr-R for the marker gene (bsr) of alphoid^{tetO} HAC.

12. Chromatin immunoprecipitation (ChIP) and real-time PCR.

ChIP with CENP-B antibody (2D8D8 and 5E6C1) was carried out according to a previously described method (Nakano et al., 2003). ChIP with antibody against EYFP (anti-Green Fluorescent Protein, Roche) was done using a modified method. Cultured cells were cross-linked in 1.0% formaldehyde. Soluble chromatin was prepared in sonication buffer (5 mM HEPES, 1.5 µM aprotinin, 10 µM leupeptin, 1 mM DTT, 0.5% SDS and 40 µM MG132) and immunoprecipitated in IP buffer (30 mM HEPES, 150 mM NaCl, 1 mM EGTA, 2 mM MgCl2, 2 mM ATP, 1.5 µM aprotinin, 10 µM leupeptin, 1 mM DTT, 0.05% SDS, 1% Triton X-100). ChIP with antibodies against CENP-A (mAN1), dimethyl histone H3 Lys4 (Upstate), trimethyl H3 Lys4 (Upstate) and trimethyl H3 Lys9 (Upstate) was done by another modified method. Cultured cells were cross-linked in 0.5% formaldehyde for 5 min, washed with TBS buffer (25 mM Tris-Cl, 137 mM NaCl, 2 mM KCl, pH 7.4), frozen in liquid nitrogen and stored at -80ºC until use. Soluble chromatin was prepared by sonication (Bioruptor sonicator, Cosmo Bio) to an average DNA size of 0.5 kb in sonication buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 1.5 µM aprotinin, 10 µM leupeptin, 1 mM DTT and 40 µM MG132), and immunoprecipitated in IP buffer (20 mM TrisHCl, pH 8.0, 600 mM NaCl, 1 mM EDTA, 0.05% SDS, 1.0% TritionX-100, 20% glycerol, 1.5 µM aprotinin, 10 µM leupeptin, 1 mM DTT and 40 µM MG132). Protein G Sepharose (Amersham) blocked with

bovine serum albumin was added, and the antibody-chromatin complex was recovered by centrifugation. The recovery ratio of the immunoprecipitated DNA from input DNA was measured by real-time PCR using the following primer sets: 5SDNA-F1 and 5SDNA-R1 for 5S ribosomal DNA, 11-10R and mCbox-4 for 11-mer of Chromosome 21 alphoid DNA (alphoidchr. 21) (Nakano et al., 2003), Sat2-F1 and Sat2-R1 for pericentromeric satellite 2 repeat (Nakano et al., 2003), tet-1 (5'-CCACTCCCTATCAG TGATAGAGAA-3') and tet-3 (5'-TCGACTTCTGTTTAG TTCTGTGCG-3') for the alphoid^{tetO} HAC, SA3 and JRN (5'- AATTCACTAGCGAATTCCC-3') for cloned alphoid DNA of a control HAC derived from synthetic alphoid 11-mer (alphoid11mer) (Ohzeki et al., 2002), bsr-F and bsr-R for the marker gene (bsr) of tetO alphoid HAC (Nakano et al., 2003), NEO1 (5'-TGGATTGCACGCAGGT TCTCCGGC-3') and NEO2 (5'-GGCATCAGAGCAGC CGATTGTCTG-3') for the marker gene (Neor) of the wt11-mer HAC.

13. Microscopy and image analysis.

Cells were transfected, fixed for 10 min with 4% PFA and mounted with VectaShied for microscopy, which was performed with a DeltaVision (Applied Precision, Issequah, WA) inverted microscope. For analysis of the intensity of various FPs on the HAC, Z-stacks were acquired using the same Z-spacing and exposure without deconvolution. We then defined a cylindrical region of interest through the stacks using the image analysis tool of Softworx and summed the intensity within this region for each image plane. The total intensity for image planes containing the HAC (Fig. 20) was normalized for background by dividing for the summed intensity above and below the HAC within the cylindrical ROI. To avoid variability associated to different levels of expression (i.e. nucleoplasmic signal), cells with similar backround intensity were selected. The analysis of signal intensities described in Chapter IV was performed with the software Image Pro Plus (Media Cybernetics, Inc), on images acquired at the same time with the same exposure. The intensities of various antibody stainings were obtained

by drawing a region of interest (ROI) around the HAC. The background intensity was measured as the average of intensity from 3 identical regions in proximity of the HAC-ROI. Cells with similar levels of background signal were selected for analysis. To normalize for the average ACA staining of endogenous centromeres (Figure 25), ROIs identical in size were used for all endogenous centromere spots as for the HAC.

III. A new type of human artificial chromosome containing synthetic sequences

1. Introduction

Artificial Chromosome technology was originally developed in *Saccharomyces cerevisiae* and later in the fission yeast *S. pombe.* Based on these early studies, it was clear that at least three chromosomal elements were required to obtain stable linear artificial chromosomes: centromeres, telomeres and origins of replication (Clarke and Carbon, 1980; Murray and Szostak, 1983). The inability to clone large, stable fragments of highly repetitive centromeric DNA represented one of the greatest obstacles for the construction of Artificial Chromosomes in human cells (Neil et al., 1990). The development of methods for cloning long arrays of alpha satellite DNA allowed the generation of *de novo* centromeres in human cells and the construction of Human Artificial Chromosomes (Harrington et al., 1997; Ikeno et al., 1998).

Human Artificial Chromosomes have been shown to behave almost identically to the endogenous chromosomes during mitosis (Tsuduki et al., 2006), and have been shown to be a powerful tool for the analysis of various chromosomal elements. The main limitation of all HACs reportedly constructed and characterized so far, was that they could not be manipulated or modified after they were obtained. A project started in our laboratory by Stefanie Kandels-Lewis and Reto Gassmann, aimed to address this limitation of artificial chromosome technology by constructing a new type of alphoid DNA array that would allow *in vitro* and *in vivo* manipulation of *de novo* constructed HACs. This new type of satellite DNA was designed to contain an array of sequences that are recognized and bound by recombinant proteins.

Scientists have widely used the selective binding of transcriptional regulators to specific DNA sequences to regulate transcription from reporter genes (Gossen and Bujard, 1992). Also, these highly specific protein-DNA interactions have been used to target proteins of interest to selected chromosomal loci (Belmont and Straight, 1998). We decided to use the *E. coli* TetRepressor protein (tetR), which binds to a sequence called the

tetOperator (tetO) for the construction of the new type of alphoid DNA array. This binding is highly specific and reversible with just nanomolar concentrations of the inducer tetracycline (Izaki et al., 1966). TetR is one of the tetracycline-resistance determinants that are widely distributed in gram-negative bacteria (Mendez et al., 1980; Klock et al, 1985). Each determinant consists of two genes - tetA and tetR - which are oriented with divergent polarity, and between them is a central regulatory region with overlapping promoter and operator sequences (Hillen and Schollmeier, 1983). The resistance protein TetA is a tetracycline/metal-proton antiporter localized in the cytoplasmic membrane, while the regulatory protein TetR is a tetracycline inducible repressor (McMurry et al, 1980). TetR binds via its helix-turn-helix motif to the two tet-operator sequences, repressing the highly regulated expression of the tetA and tetR genes (Hillen and Schollmeier, 1983). In fact, the expression of TetR occurs before the expression of the resistance protein TetA and only the binding of a tetracycline-Mg^{2+} complex to the repressor causes a conformational change towards a non-DNA binding conformation, and induces transcriptional activation (Figure 11).

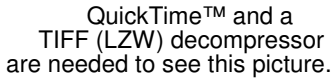


Figure 11. Complex between TetR and the 15 bp tetO sequence (adapted from Orth et al Nat Struct Biol 7:215, 2000). Binding of MgTc to the TetR induces a major swing in helices 1 to 3 (shown in blue and responsible for contact with the DNA). As a result the affinity of the TetR-MgTc complex is lost.

2. Construction and characterization of a human artificial chromosome with a synthetic alphoid DNA

2.1 Cloning a partially synthetic alphoid DNA array

The work described in this section was performed in part by Reto Gassmann in the laboratory of Prof. W. Earnshaw, and in part by Vladimir Noskov in the laboratory of Dr. Vladimir Larionov, NIH Bethesda-MA, USA.

To obtain a Human Artificial Chromosome (HAC) suitable for manipulation, a novel artificial alphoid dimer was designed by Bill Earnshaw. One half of the alphoid dimer was a monomer subcloned from the chromosome 17 alphoid 16-mer Higher Order Repeat (Waye and Willard, 1986). This was linked to a completely synthetic alphoid monomer based on a published consensus sequence for α-satellite DNA (Vissel and Choo, 1991). The natural monomer contains a CENP-B binding motif (CENP-B box, (Masumoto et al., 1989), which was replaced in the synthetic monomer with the 42 bp tetracycline operator (tetO) sequence (Fig. 12a). Since the TetO is bigger than the CENP-B box, 25% of the resulting synthetic monomer contained no alphoid sequences.

HAC formation in HT1080 cells requires input naked alphoid DNA of at least 30 kb for functional CENP-A core assembly (Okamoto et al., 2007). The artificial alphoid^{tetO} dimer was cloned conventionally to obtain an array of 3.5 kb. To further extend the array, rolling circle amplification was used to obtain a sequence of approximately 10 kb. The final extension to an array of about 50 kb was achieved by transformation-associated recombination (TAR) cloning in yeast (Fig. 12b) (Ebersole et al., 2005)**.** A BAC vector was used for the final cloning of the 50 kB of alphoid^{tetO} dimeric repeat. The BAC vector - BAC32-2mer(tetO) - contained Yeast Artificial Chromosome elements for selection of recombinants in yeast, and mini-F plasmid elements for stable

and copy-number regulated amplification in bacteria. For selection in mammalian cells, the BAC contained the Bsr gene that confers resistance to the drug Blasticidin.

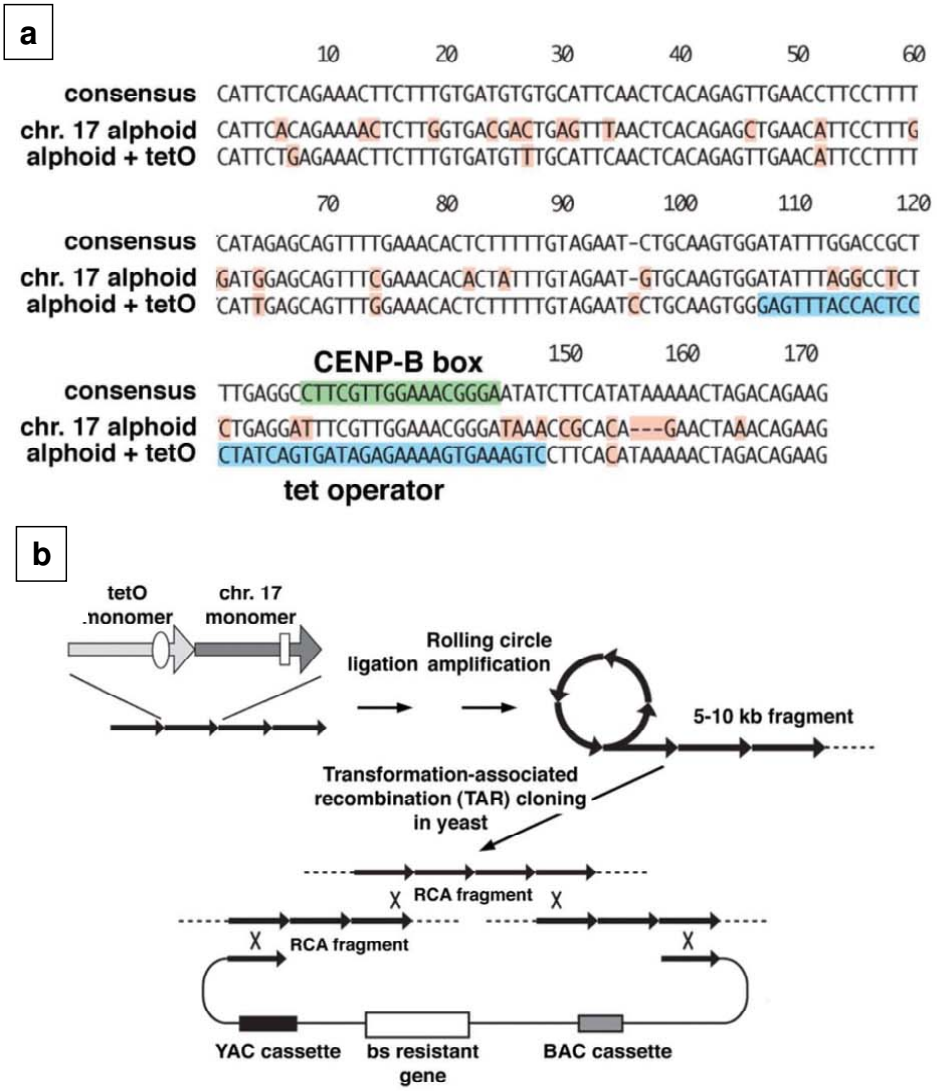


Figure 12. Construction of a synthetic alphoid^{tetO} array.
(a) Schematic of the synthetic alphoid^{tetO} dimer. The first monomer sequence was synthesized *in vitro* based on a published consensus for alphoid DNA, and a tetO sequence was inserted in place of the CENP-B box. The second monomer was cloned from the Chromosome 17 alphoid repeat, and contains a CENP-B box. **(b)** The alphoid dimer was extended to a sequence of approx. 50kb by rolling circle amplification followed by TAR cloning. Work in (b) was designed by Vladimir Larionov and performed by Vladimir Noskov.

2.2 De novo formation of an alphoid^{tetO} HAC in HT1080 cells

This work was entirely performed by Megumi Nakano and Hiroshi Masumoto in the laboratory of Dr. Vladimir Larionov, NIH Bethesda-MA, USA.

To assess the ability of the synthetic alphoid^{tetO} DNA to form an artificial chromosome, BAC32-2mer(tetO) was introduced by electroporation in human HT1080 cells. After transfection, cells were selected for resistance to blasticidin and several cell lines were isolated for further analysis. FISH with probes specific for the alphoid^{tetO} dimer or for the BAC backbone revealed that 2 of 46 transformant cell lines analyzed carried an artificial chromosome (Fig. 13a and Table 1A). The HAC formation efficiency for the alphoid^{tetO} DNA was 4.3%, and the fractions of HAC-containing cells were 35.7% and 28.6% for two cell lines analyzed (AB2.2.18.21 and AB2.5.4.19, Table 1B). The HAC- formation efficiency and fraction of HAC-containing cells were both lower compared to that obtained with a 60 Kb natural type 1 alphoid DNA cloned from chromosome 21 (respectively 30% and >50%, α21-I, Table 1). No host chromosomal DNA could be detected by inter- and intra-Alu PCR probes on the alphoid^{tetO} HAC (Figure 13a).

Sub-cloning yielded several cell lines containing one copy of the alphoid^{tetO} HAC. Regardless of the lower formation efficiency, the alphoid^{tetO} HAC in AB2.2.18.21 and AB2.5.4.19 cell lines showed very high mitotic stability in absence of drug selection (loss rate per division = 0.0024, or 0.0054 respectively, Table 1B).

Table 1A Efficiency of HAC formation following transfection with alphoid BACs

Introduced DNA	Analyzed cell lines	No. of cell lines: either HAC or integration	
		HAC	Host chromosome integration
Wild type 11.32	41	12(29.3%)	29(70.7%)
BAC32-2mer(tetO)	46	2 (4.3%)	40(87.0%)

□4(8.7%) cell lines showed extra minichromosome signals containing host chromosomal fragments

Table 1B Frequency of BAC32-2mer(tetO) derived HACs

		Copy number of BAC32-2mer(tetO)		ratio of cells: either HAC or integration		
Clone	loss rate	alphoid^{tetO} dimer	bsr	HAC	centromere	arm
AB2.2.18				35.7%	0	64.3%
AB2.2.18.21*	0.0024	47.6	46.5	100%	0	0
AB2.5.4				28.6%	71.4%	0
AB2.5.4.19**	0.0054	16.8	15.8	100%	0	0

□ suclone of AB2.2.18
** subclone of AB2.5

Isolation of HACs was performed by Megumi Nakano in the labs of Hiroshi Masumoto and Vladimir Larionov.

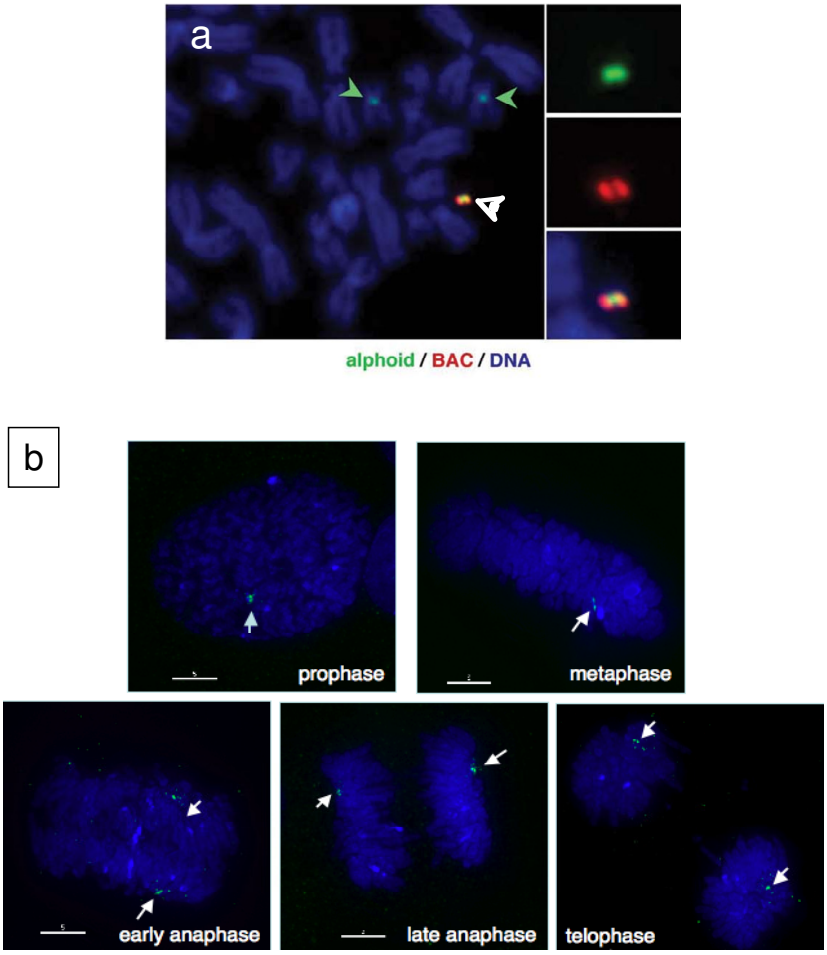


Figure 13. FISH analysis on the AB2.2.18.21 cell line containing a alphoid^{tetO} HAC.
(a) FISH with Chromosome 17 alphoid probe (green) or a BAC probe (red). The HAC stains positively for both the alphoid and the vector BAC-backbone probes (white arrowhead). Chromosomes 17 alphoid regions are indicated by green arrowheads. **(b)** FISH on AB2.2.18.21 cells with a BAC-probe (green). Micrographs show cells at different stages of mitosis. White arrows point to the HAC.
Work in (a) was performed by Megumi Nakano.

2.3. Analysis of the chromatin and kinetochore proteins associated to the alphoid^{tetO} HAC

The HAC formation efficiency shown by the synthetic alphoid^{tetO} was lower than that obtained with endogenous alphoid DNA. Nevertheless, the alphoid^{tetO} HAC showed a mitotic stability similar to HACs obtained with natural alphoid repeats. I used the AB2.2.18.21 cell line for the further analysis of mitotic behaviour of the alphoid^{tetO} HAC, and for the manipulation of the synthetic alphoid^{tetO} array. The HAC in this cell line demonstrated high mitotic stability, with a loss rate of 0.0024, and contained no detectable host DNA (not shown). Indeed, Fluorescence *in situ* Hybridization performed with a BAC probe showed that the alphoid^{tetO} HAC segregated normally in mitosis (Figure 13b).

To further study the composition of the kinetochore assembled on the HAC, I used FISH-coupled immunostaining (ImmunoFISH) on AB2.2.18.21 cells. To test the association of kinetochore proteins with the alphoid^{tetO} HAC, I used antibodies against CENP-A, CENP-B and CENP-H (Warburton et al., 1997; Sugata et al., 2000). In addition, the association of the chromosomal passenger complex, a key factor for proper mitotic progression (Vagnarelli and Earnshaw, 2004), was analyzed by staining with an anti-INCENP antibody. Incubations with different antibodies were followed by FISH analysis with a probe that hybridizes specifically to the alphoid^{tetO} HAC. The results of this analysis revealed that the centromere histone-variant CENP-A, and constitutive kinetochore components CENP-C and CENP-H, assembled normally on the alphoid^{tetO} DNA (Figure 14a-c). Previous RNAi experiments revealed that the centromere localization of hMis12 occurs upstream of that of CENP-H in human cells (Goshima et al., 2003; Kline et al., 2006). Therefore also hMis12 is presumably associated to the HAC in mitosis. The staining for the passenger protein, INCENP, showed a signal stretching between the two BAC-positive HAC sisters aligned on a metaphase plate. This confirmed the association of the passenger complex to the alphoid^{tetO} HAC in mitosis (Figure 14d).

In conclusion, these results demonstrated that the alphoid^{tetO} HAC contains an active centromere that is able to recruit a functional complement of kinetochore proteins.

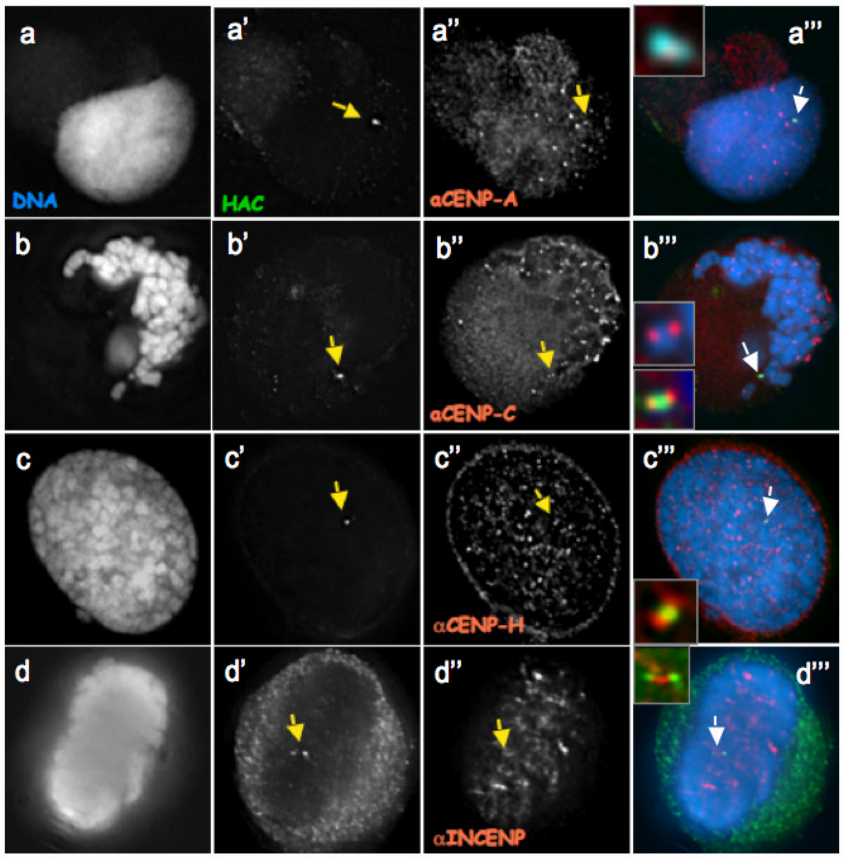


Figure 14. ImmunoFISH analysis of the recruitment of centromere/kinetochore components. FISH with a BAC-probe (a'-d') was coupled to immunostaining with antibodies for CENP-A (a''), CENP-C (b'') CENP-H (c'') and INCENP (d''). The HAC recruited all proteins analyzed (arrows) and it was also possible to observe INCENP stretching between the two sister HACs aligned on a metaphase plate (d'').

2.4. Analysis of the alphoid^{tetO} chromatin by ImmunoFISH on extended chromatin fibers

Extended chromatin fibers have been used to analyze the distribution of histone tail modifications over defined regions of DNA. In human and fly, studies on fibres obtained from centromeric regions revealed that the inner part of alphoid satellite DNA is usually dimethylated on lysine 4 of histone3 (H3K4me2). This modification has been extensively associated to regions of chromatin that are competent for transcriptional activity.

To study the distribution of specific histone modifications on the synthetic alphoid^{tetO} DNA of the HAC, I performed immunoFISH on extended fibres obtained from AB2.2.18.21 cells. I used a BAC probe to locate chromatin fibres belonging to the HAC and antibodies for the dimethylated Lysine 4 (H3K4me2) and trymethylated Lysine 9 (H3K9me3) of histone H3, together with an antibody for the centromeric histone-variant CENP-A. This analysis revealed an interspersed arrangement of CENP-A and K4me2-modified histone H3 clusters (Figure 15a). This distribution is similar to the alternate pattern of these nucleosomes clusters at endogenous centromeres (Sullivan and Karpen, 2004). Interestingly, CENP-A nucleosomes clusters were interspersed also with H3-nucleosomes that were trimethylated on Lysine 9 (H3K9me3, Figure 15b). This result reveals that this type of histone modification is also present at the synthetic alphoid^{tetO} HAC array.

Another important observation from this study was that the alphoid sequences, defined as CENP-A-enriched regions, were interspersed with BAC positive regions that presumably did not contain alphoid DNA. This confirmed independent conclusion from pulsed field gel analysis (not shown) indicating that the original alphoid^{tetO} array was multimerized during the formation of the artificial chromosome in HT1080 cells.

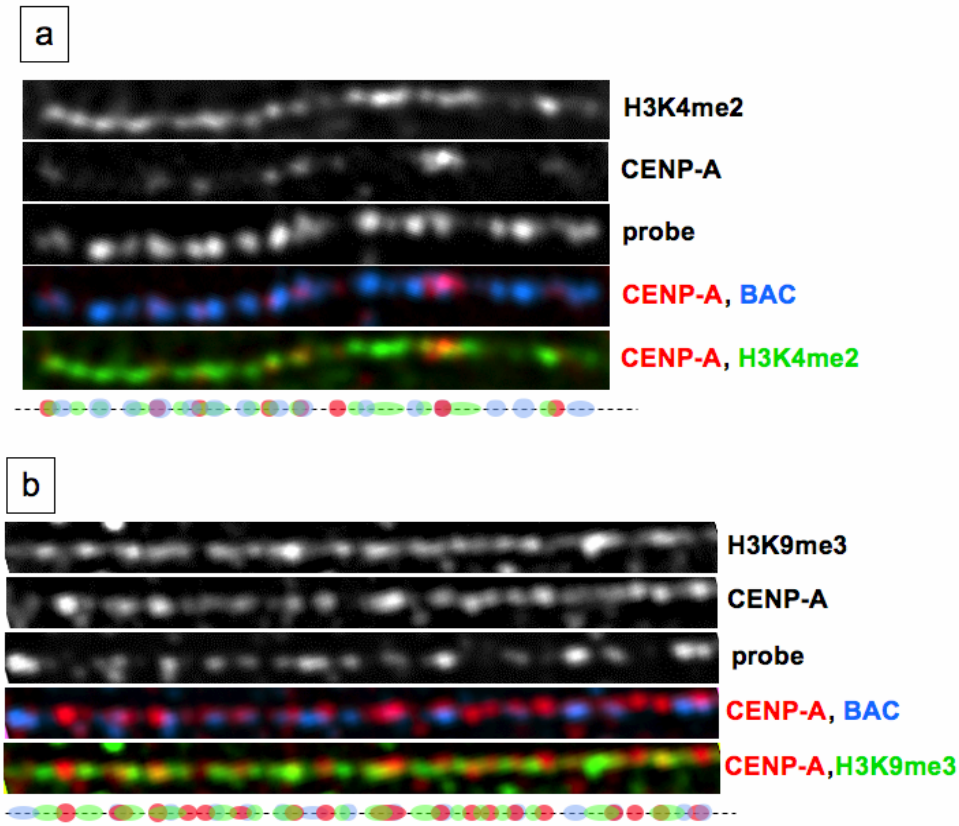


Figure 15. ImmunoFISH on extended chromatin fibers.
Chromatin fibers were obtained from asyncronous AB2.2.18.21 cells as described in Materials and Methods (Chapter II). A BAC probe (blue) was used for the FISH to localize HAC-fibers, and antibodies for H3K4me2 (a) and H3K9me3 (b) were used in combination with an anti-CENP-A antibody (in red). To facilitate the analysis, the clusters positive for the different antibodies or for the FISH probe have been aligned at the bottom of the micrographs.

2.5. Analysis of the chromatin structure by ChIP

This work was entirely performed by Megumi Nakano, Dr Larionov's laboratory, NIH Bethesda-MA, USA.

Chromatin Immunoprecipitation (ChIP) was used as a second approach to analyze the type of chromatin assembled on the alphoid^{tetO} HAC. This analysis allowed us to determine the amount and distribution of the centromere-specific histone variant CENP-A, of CENP B, and of various histone modifications associated with "closed" or "open" types of chromatin.

Cell lines AB2.5.4.19 and AB2.2.18.21, both containing an artificial chromosome with no detectable host DNA, were used for this analysis. Oligonucleotides specific for the alphoid^{tetO} sequences or the Bsr gene were used for quantitative PCR (qPCR) to detected the enrichment of HAC-specific sequences. For normalization, oligonucleotides for endogenous ribosomal DNA (rDNA), γ-satellite DNA (Sat2) and Chromosome 21 alphoid DNA (11-mer) were used for qPCR.

ChIP confirmed that the synthetic alphoid^{tetO} repeats are located within the active kinetochore of the HAC. Immunoprecipitation with antibodies for CENP-A and CENP-B yielded enrichment of the alphoid^{tetO} repeats comparable to that seen for control endogenous centromeres (figure 16). Furthermore, the enrichment of these proteins on alphoid^{tetO} sequences was similar to what is found with HACs containing classical α-satellite DNA (alphoid$^{11\text{-}mer}$ HAC).

H3K4me2, a marker for transcriptionally competent or neutral chromatin (Santos-Rosa et al., 2002; Schneider et al., 2004), has recently been proposed to be a marker for centromeric chromatin (Sullivan and Karpen, 2004; Schueler and Sullivan, 2006). Indeed, regions containing H3K4me2 tended to alternate with regions containing CENP-A in extended HAC fibers (Fig. 15a). H3K4me2 was enriched on alphoid^{tetO} repeats at levels significantly above those on chromosome 21 alphoid DNA or the alphoid^{11mer} control HAC (Fig. 16). This result suggests that the alphoid^{tetO} array is enriched of a slightly more transcriptionally competent chromatin compared

to native centromeres. However, because the alphoid^{tetO} and the control HACs have similar stability, these subtle differences must not interfere with kinetochore function.

*ChIP analysis revealed high levels of trimethylated histone H3 Lys4 (H3K4me3), a marker for transcriptionally active chromatin (Jenuwein and Allis, 2001; Santos-Rosa et al., 2002; Schneider et al., 2004), on the marker gene of the alphoid^{tetO} HAC. Much lower H3K4me3 levels were found on the alphoid^{tetO} repeat itself (Fig. 16). Consistent with this, trimethylated histone H3 Lys9 (H3K9me3), a marker for silent chromatin (Peters et al., 2003; Guenatri et al., 2004), was associated with the alphoid^{tetO} array (Fig. 16). This result was consistent with that observed on extended chromatin fibers, where H3K9me3 was alternated with CENP-A-nucleosome clusters (Fig. 15b).

Together, these data confirm the overall similarities between the alphoid^{tetO} and endogenous centromeric alphoid chromatin.

**quoted from the paper in Appendix.*

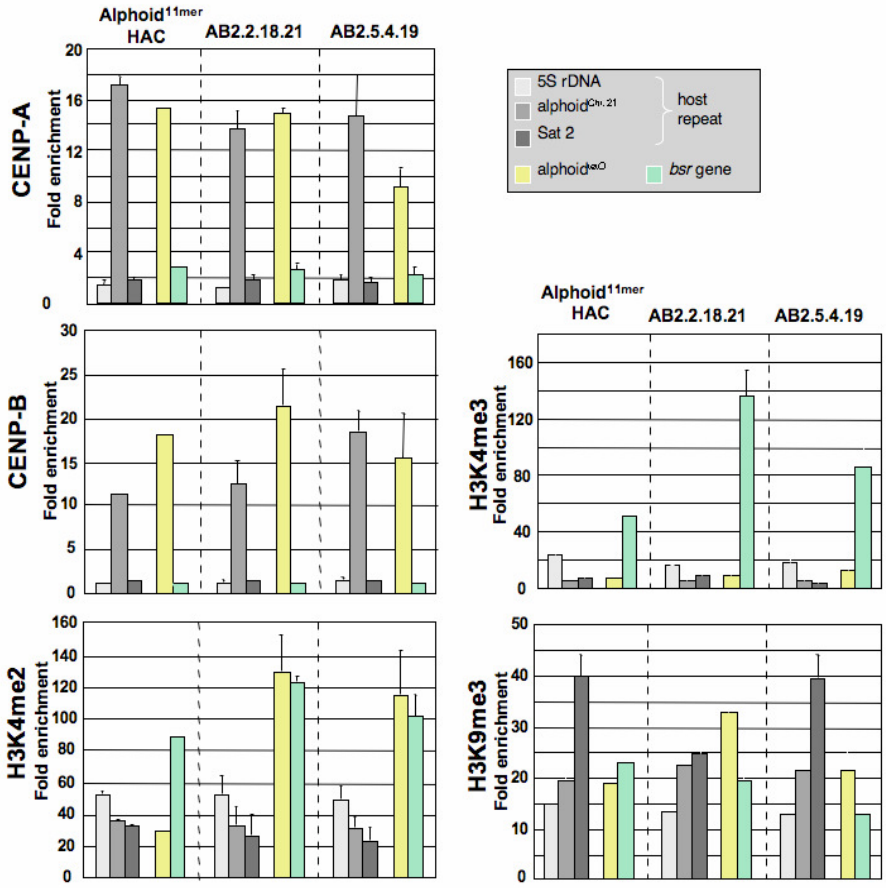


Figure 16. ChIP analysis of the chromatin associated to the HAC.

Antibodies for CENP-A, CENP-B, H3K4me2, H3K4me3 and H3K9me3 were used for ChIP on two cell lines containing an alphoid^{tetO} HAC AB2.2.18.21 and AB2.5.4.19, and a cell line containing a HAC constructed with 11-mer alphoid DNA cloned from chromosome 21 - alphoid^{11mer}HAC. The immunoprecipitated DNA was quantified using oligonucleotides for the HAC tetO sequence the blasticidin gene (Bsr), ribosomal DNA (rDNA), alphoid from chromosome 21 (11-mer) and g-satellite DNA (Sat2). Experiments performed by Megumi Nakano.

3. Targeting of chromatin modifying enzymes to the alphoid^{tetO} HAC

3.1 Tetracycline repressor and centromere proteins associate with alphoid^{tetO} sequences in the HAC kinetochore

To test the ability of tetR to bind the array of tetO sequences embedded in the HAC, I transfected AB2.2.18.21 cells with different constructs expressing combinations of fluorescent proteins (FP) and tetR. The first construct tested was a combination of the monomeric Red Fluorescent Protein (mRFP) fused to the N-terminus of a class B TetRepressor (Waters et al., 1983; Unger et al., 1984). mRFP-TetR showed, at low-medium expression levels, a diffuse nuclear localization and a single bright spot. This bright spot co-localized with inner kinetochore proteins including CENP-B, CENP-C, CENP-H and with ACA, an anti-immune antigene that binds to CENP proteins (Earnshaw and Migeon, 1985) (Figure. 17a-c).

It was considered that the binding of a high number of exogenous proteins might somehow disrupt the functionality of the alphoid^{tetO} HAC. However, the ability of mRFP-bound HAC to recruit CENP proteins disproved this hypothesis, also because TetR itself is able to dimerize therefore increasing its isteric hindrance. Furthermore, I could observe cells with mRFP-coated HACs segregating normally in telophase (Figure 17d).

In another construct I linked the Yellow Fluorescent Protein (YFP) to the C-terminus of a slightly different TetR protein (pTet-On/Off, Invitrogen), which however contains the same DNA-binding region as the mRFP-TetR construct. When this new construct - TetR:YFP – was expressed in AB2.2.18.21 cells, it localized similarly to mRFP-TetR, with a diffuse nuclear signal and a bright spot. Also in this case, the TetR:YFP aggregate co-localized with kinetochore core proteins and images of transfected mitotic cells showed that TetR:YFP-coated HACs aligned normally on the

metaphase plate, with the stretched pair of YFP-positive HAC sisters co-localizing with CENP-C (Figure 17e).

In conclusion, the tetO sequences of the alphoid^{tetO} HAC were accessible to the binding of exogenously expressed constructs. FP-linked TetR proteins specifically localized to the HAC and did not disrupt the binding of kinetochore –associated CENP-proteins or the mitotic segregation of the HAC.

Figure 17. Binding of TetR-fusions to the alphoid^{tetO} DNA

(a-d) Cells were transfected with mRFP:TetR and fixed for staining 48 hours post-transfection. The construct localized diffusely in the nucleus with a bright spot (a-c, d and red) that co-localized with antibodies against CENP-B (a'), CENP-C (b') and CENP-H (c') (arrows indicate the spots). **(e)** TetR:YFP binds to the alphoid^{tetO} HAC in mitosis (e green and arrow). The targeted HAC aligned at the metaphase plate and co-localized with an anti-CENP-C antibody (e').

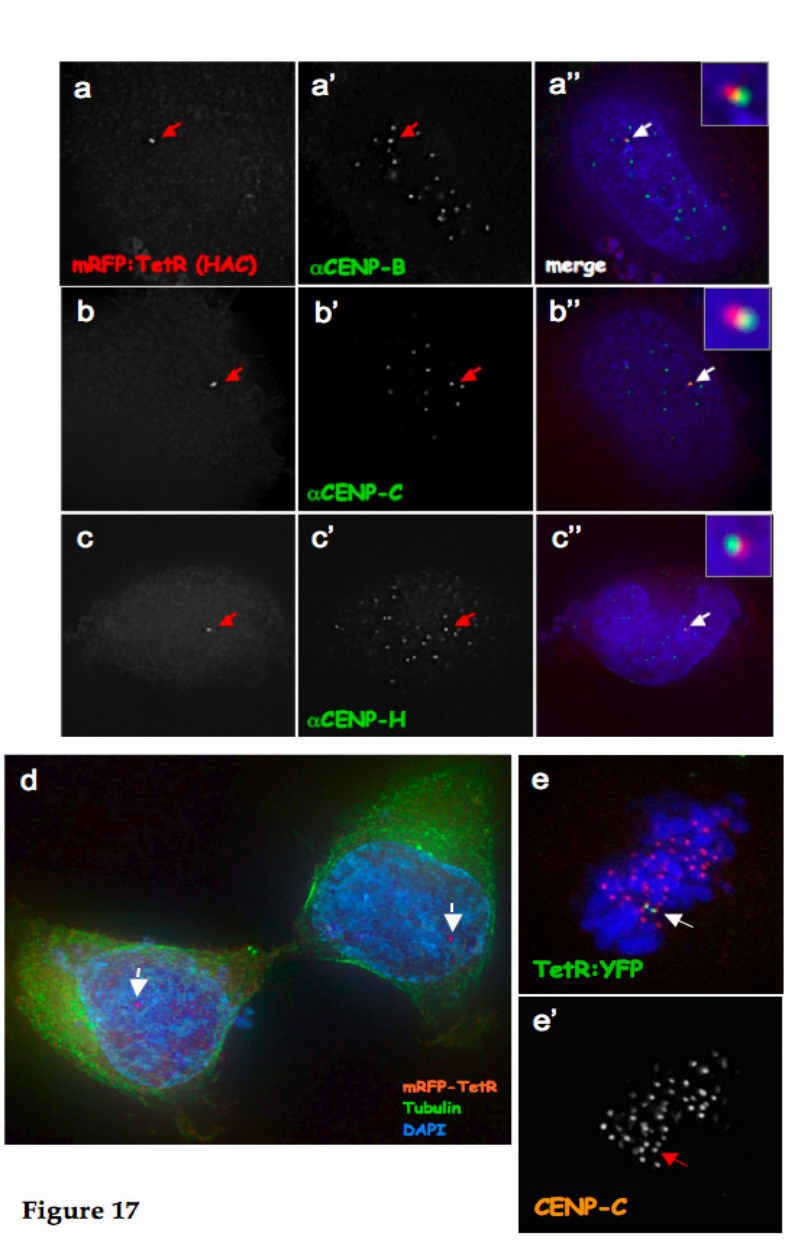

a
a'
a''
mRFP:TetR (HAC)
αCENP-B
merge
b
b'
b''
αCENP-C
c
c'
c''
αCENP-H
d
mRFP-TetR
Tubulin
DAPI
e
TetR:YFP
e'
CENP-C

Figure 17

3.2 Binding of the tTA transactivator can induce alphoid^{tetO} HAC loss

The single cell analysis was performed by me, whereas the population analsys of loss rate by qPCR was performed by Megumi Nakano.

The accessibility to TetR of the alphoid^{tetO} HAC suggested that it might be possible to directly test the effects of targeting chromatin modifiers on kinetochore function. A strategy was designed to modify the synthetic alphoid chromatin in order to disrupt centromeric functionalities, such as the ability to incorporate the centromere-specific histone variant CENP-A. Centromeric chromatin has been recently defined as transcriptionally competent (reviewed in Schueler and Sullivan, 2006), and recent studies on rice and human neocentromeres suggest that centromeres can contain actively transcribed genes (Saffery et al., 2003; Nagaki et al., 2004; Yan et al., 2006). In contrast, heterochromatin has been widely characterized as flanking the centromere region (pericentromeres), and found to be important for its maintenance (Guenatri et al., 2004; Nakashima et al., 2005; Lam et al., 2006).

To test the compatibility of euchromatin with centromere functions, I constructed a targeting protein containing a TetR and a transcription activation domain, to be transfected into cells the alphoid^{tetO} HAC. For targeting, I made tTA, a fusion between TetR and Herpes Simplex VP16 transcription activation domain. The tTA is widely used to regulate the expression of reporter genes. To test whether the functional disruption of centromere activity was due to transcriptional activity, I used several tTA variants that have diminished transcriptional activation efficiency. In this study, I used tTA3 and tTA4, which retain respectively 39% and 14% of the wild-type activity (assayed on a tTA-responsive reporter gene, not shown). As a transcriptional repressor I have used tTS, which is a fusion of the KRAB repression domain of a mouse kidney protein (Kid1)(Witzgall et al., 1994) and a hybrid TetR protein. Mitotic stability of the alphoid^{tetO} HAC was used to analyze the disruption of centromeric function.

*Targeting of tTA to the alphoid^{tetO} HAC produced a mosaic response in which the HAC kinetochore was inactivated in some cells, but not in others. In a "cell-by-cell" assay, I transfected cells bearing the alphoid^{tetO} HAC with various plasmids, killed the non-transfected cells with puromycin and scored the percentage of nuclei bearing 0, 1 or 2 copies of the HAC (detected by FISH with the BAC probe) after 11-12 days of culture (Figure 18a). To control for effects of the transfection procedure on HAC stability, all values were normalized to those for cells transfected with plasmid carrying only a puromycin-resistance gene.

Transfection of cells with plasmids expressing tetR-EYFP had essentially no effect on HAC stability (Fig. 18b). In contrast, tTA expression caused a reproducible increase in the population of cells lacking the HAC in the nucleus (Fig. 18b, n=3-5). Transfection with tTA3 (39% as active as tTA in promoting transcription) or tTA4 (14% activity) also caused significant, albeit reduced, levels of HAC loss. The tTA had no effect on HAC stability if cells were grown in the presence of doxycycline.

To confirm the cytological analysis of the tTA targeting, Megumi Nakano used a quantitative population assay to quantify the loss-rate of the targeted alphoid^{tetO} HAC. AB2.2.18.21 cells were infected with retroviral vectors coding for tTA and controls, and the copy number of alphoid^{tetO} dimer in selected clones was quantified thirty days later by quantitative PCR (qPCR) (Figure 19a). Cells infected with control vectors expressing the marker gene or tetR alone showed no significant decrease in HAC stability (Fig. 19b). Cultures expressing the tTA showed a 6-fold increase in the HAC loss (27% decrease in HAC content, see M&M for the formula used) (Figure 19b). No significant HAC loss was observed in parallel cultures grown in the presence of doxycycline (Fig. 19b striped bars).

**Quoted from the paper in Appendix.*

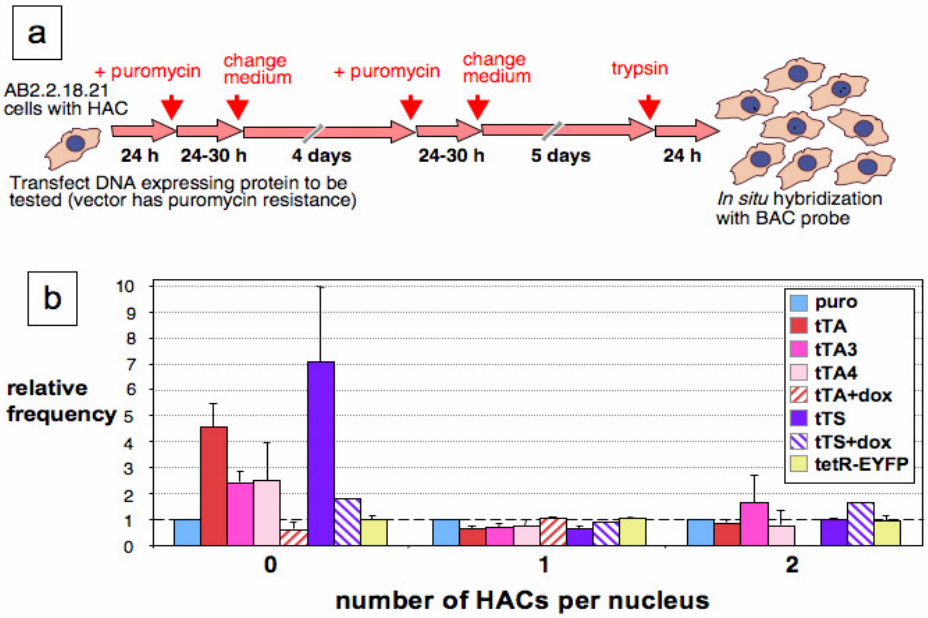


Figure 18. Cytological analysis of HAC mitotic stability

(a) Schematic of the experimental procedure. Cells were transfected with different targeting constructs and selected with puromycin 24 hours post-transfection. Another step of drug-selection was done at 5 days, and cells were cultured for 12 days before fixation. **(b)** FISH analysis of the number of HACs (0, 1, 2) per nucleus in cell populations transfected with the indicated constructs (see legend). The fraction of nuclei is calculated as relative to control Puro experiment (light blue) (relative frequency).

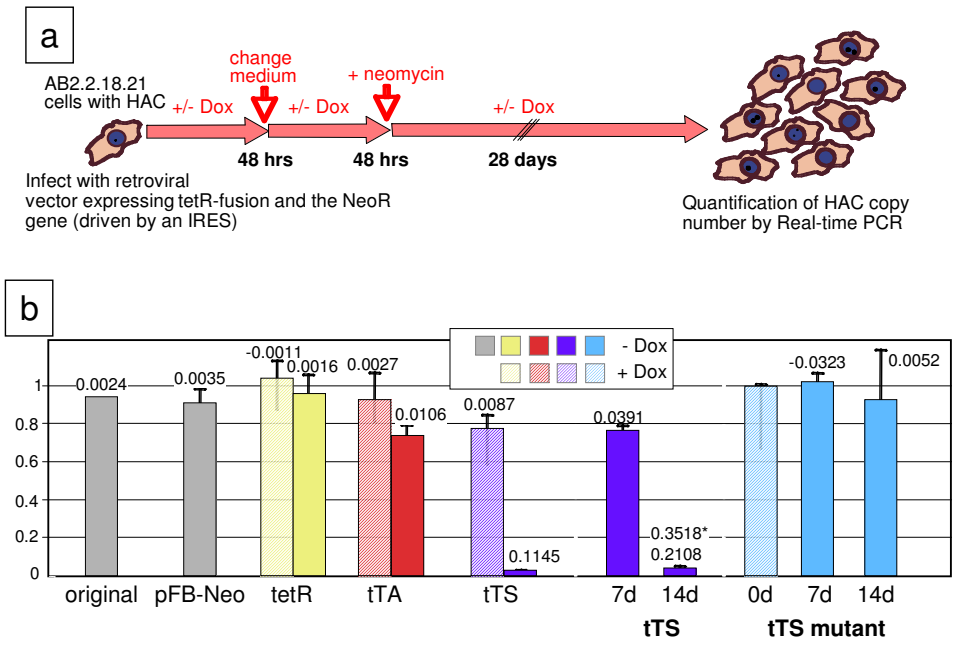


Figure 19. Population analysis HAC mitotic stability

(a) Schematic of the experiment. Cells were transfected with several targeting constructs in the presence or not of Doxycycline and selected with neomycin 96 hours post-transfection. Cells were cultured for a total of 32 days. Samples were collected at 7, 14 and 30 days post-transfection. **(b)** Analysis of HAC copy-number by qPCR. The values represent an average of qPCR amplifications using oligonucleotides for tetO and Bsr HAC sequences. These values are normalized for the control (untransfected) value (=1). Striped bars: control cultures grown in the presence of doxycycline (no HAC binding).

All data are from Megumi Nakano.

To demonstrate that the tTA was transcriptionally active after binding the DNA, the levels of mRNA transcripts were quantified by qPCR (analysis of Megumi Nakano). tTA expression caused a ~2-fold increase in the very low level of alphoid^{tetO} transcripts, but had no effect on the much higher levels of transcription from the *bsr* gene (Fig. 20a', a'').

In contrast with the mild effect observed on mitotic stability by tTA, the examination of the HAC chromatin failed to reveal significant structural differences detected by ChIP with antibodies to H3K4me2 or H3K4me3 (data not shown). This is most likely because tTA binding to the HAC elicits a mosaic response, in which a minority of cells respond by increasing transcription, and destabilizing the HAC (Figs. 18b, 19b and 20a). Since the HAC appears to resist effects caused by tTA binding in most cells, this could explain why the chromatin changes analyzed by ChIP remain below our limit of detection.

> *These experiments demonstrate that the alphoid^{tetO} HAC kinetochore can be inactivated in a subset of cells by targeting its chromatin with a transcriptional activator. If, indeed, kinetochore inactivation is induced by transcription of the alphoid^{tetO} array, this result might be similar to that observed in budding yeast, where strong transcriptional bombardment can inactivate a conditional centromere (Hill and Bloom, 1987).

* *Quoted from the paper in Appendix.*

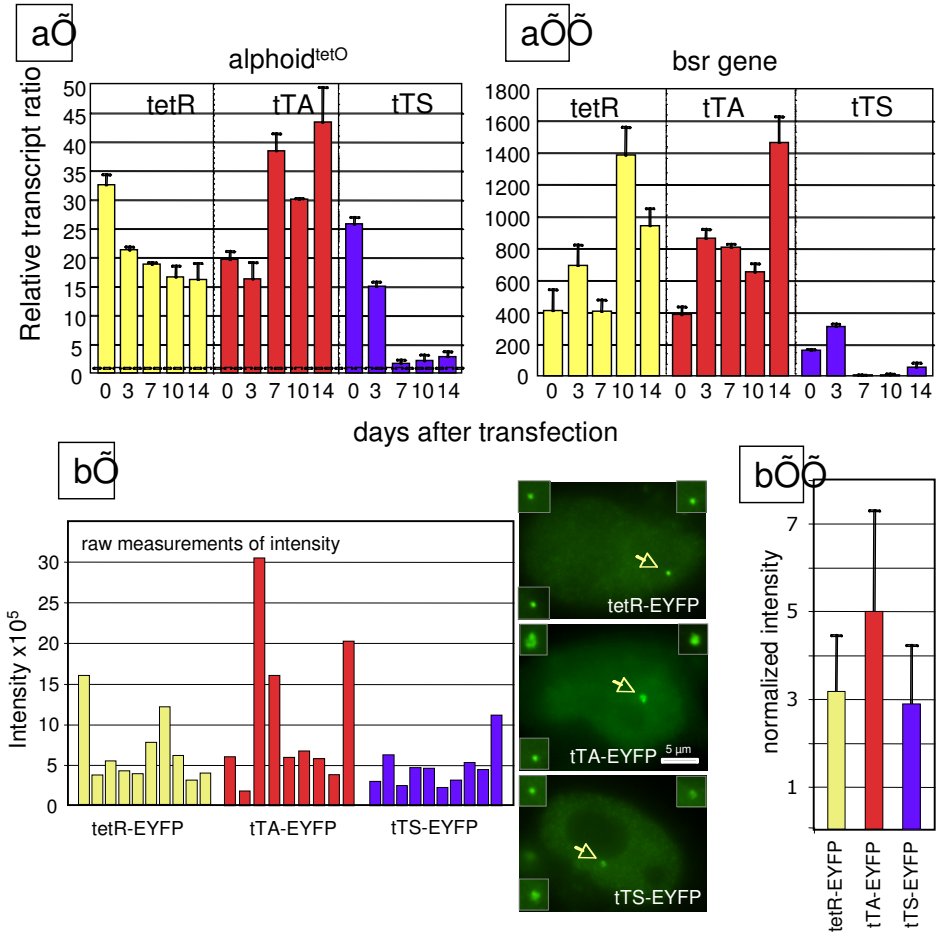


Figure 20. Activity and binding of TetR-constructs

(a) The enzymatic activity of TetR:YFP, tTA:YFP and tTS:YFP were assayed by Reverse-Transcription PCR.The level of transcription associated to alphoid^{tetO} array (a') and to the Bsr gene (a'') was analyzed at different time-points (x-axis) after transfection. tTA caused a >2 fold increase of transcription compared to control TetR:YFP. In contrast, tTS decreased transcription to background levels. Data from experiments performed by Megumi Nakano. **(b)** Measurements of the YFP signal associated to the HAC 48 hours after transfection with tetR:YFP, tTA:YFP and tTS:YFP. Raw intensities are similar among the constructs (b'). However, in some cases tTA showed a marked amount of signal, distributed over a bigger region (see micrograph). Remarkably, despite the severe effect on the HAC mitotic stability, the average of intensities of tTS-binding are similar to TetR (b'').

3.3 Binding of a transcriptional silencer induces dramatic alphoid^{tetO} HAC loss

Facultative heterochromatin is usually found at developmentally regulated loci, where the chromatin state can change in response to cellular signals and gene activity. One feature of heterochromatin is to propagate and repress transcription from flanking regions, in a sequence-independent manner (Demerec, 1940; Hartmann-Goldstein, 1967). Although several studies showed that in certain circumstances heterochromatin can be required for activation of gene expression (Lu et al., 2000; Yasuhara and Wakimoto, 2006), epigenetic gene silencing has become synonymous of heterochromatization. In fact, heterochromatin mediated chromatin-modifying activities may prevent access of the DNA to the transcriptional machinery (Yamada et al., 2005).

Following the demonstration that euchromatin can partially disrupt centromere functions, I turned to analyze whether heterochromatin has the same effect. To trigger formation of heterochromatin, I used the transcriptional repressor tTS in a fusion with YFP (tTS:YFP). tTS is a combination of tetR with the Kruppel-associated box (KRAB)-silencing domain of Kid-1 (kidney, ischemia, development protein, Freundlieb et al., 1999; Witzgall et al., 1994). tTS has been used in the past to study mechanisms of transcriptional repression and is now also used for tightly regulated expression of genes of interest. The fusion with YFP allows a direct visualization of the targeting construct in transfected cells.

For the cytological analysis of alphoid^{tetO} HAC mitotic stability, the same protocol applied for tTA inactivation was used. Cells were transfected with control TetR:YFP and tTS:YFP, selected with puromycin 1 and 5 days after transfection and cultured for 12 days. Samples of the transfected cells were then processed for FISH analysis and the number of alphoid^{tetO} HACs retained in the nucleus was counted. This cell-by-cell analysis revealed a much stronger inactivation effect caused by the tTS compared to the tTA. In

transfected cells that were cultured for 12 days, there was a 6-fold increase (relative frequency, compare with 4.5 fold of tTA) in the fraction of nuclei that had lost the alphoid^{tetO} HAC relative to control (Figure 18b).

In the analysis by qPCR of alphoid^{tetO} HAC copy-number under targeting conditions, Dr. Nakano demonstrated that tTS induces a highly penetrant destabilization of the HAC (Figs. 19b).

> *Unlike the variable and weak effects seen following expression of tTA, tTS-transfection caused a 72-fold increase in the rate of HAC loss (97% decrease in HAC content) 30 days after infection with retroviral vectors expressing the construct, but HAC loss was essentially complete by 14 days of tTS expression (Figure 19b, only time-points at 7 and 14 days are shown). Expression of a tTS point mutant (tTS^{mut}) that is unable to bind the corepressor KAP-1 (KRAB-associated protein-1) (Agata et al., 1999; Matsuda et al., 2001), had no effect on alphoid^{tetO} HAC stability even after 14 days (Fig. 19b). Thus, destabilization of the HAC by the tTS apparently occurs via the KAP-1 pathway (KAP1 is a KRAB-binding protein - see next chapter).
>
> (**Quoted from the paper in Appendix)*

In control experiments, I determined that the differential effects of tetR-EYFP, tTA and tTS:YFP on HAC stability could not simply be explained by differences in the binding of these proteins to the alphoid^{tetO} array. After the construction of tTA:YFP, I analyzed the fluorescence of the HAC-bound fraction (normalized for the unbound nucleoplasmic signal) of the different YFP-linked targeting constructs. There were no significant difference between the averages of the different intensities of fluorescence of the bound constructs (Fig. 20b''). However, in some cells, HACs with bound tTA-EYFP appeared to be larger, and the raw fluorescence intensity measures revealed a higher amount of HAC-bound protein (Fig. 20b'). Importantly, the tTS, which was a much stronger HAC inactivator compared to tTA, bound to alphoid^{tetO} HAC at levels comparable to the tetR, which had no observable effect on HAC stability.

4. Analysis of the mechanism of HAC loss

4.1 The tTS disrupts CENP-A kinetochore chromatin

This work was entirely performed by Megumi Nakano, NIH Bethesda-MA, USA.

The severe loss of mitotic stability of the alphoid^{tetO} HAC following tTS targeting might be a consequence of variations in the underlying chromatin. To analyze possible effects on the chromatin in the targeted region, ChIP was used with antibodies for histone H3 modifications dimethyl-lysine 4 (H3K4me2), trimethyl-lysine 4 (H3K4me3) and trimethyl-lysine 9 (H3K9me3), and the centromeric histone variant CENP-A. The HAC alphoid^{tetO} array and Bsr gene were analyzed, together with host chromosome 21 alphoid DNA (11-mer), ribosomal DNA (rDNA) and γ-satellite DNA (Sat2).

Comparing ChIP data collected 7 and 14 days post-transfection revealed that tTS binding caused a rapid loss of H3K4me2 and somewhat slower decrease in CENP-A levels on the alphoid^{tetO} array (Fig. 21a-b). In particular, CENP-A seemed to partially spread over the Bsr marker gene at 7 days, before returning to near-background levels at the later time-point. Levels of the H3K4me3 modification were also decreased (Fig. 21c). Surprisingly, the amount of H3K9me3 modification was much higher on the alphoid^{tetO} array after tTS binding at 7 days, but dramatically dropped at 14 days (Fig. 21d). tTS binding also reduced H3K4me2 and H3K4me3 levels and increased H3K9me3 levels on the marker gene (bsr). In controls, binding of tetR to the alphoid^{tetO} HAC caused no detectable changes in the HAC chromatin.

The closed chromatin structure induced by tTS binding appeared to spread laterally onto the BAC-vector sequences flanking the alphoid^{tetO} arrays. Furthermore, although only 4% of the HAC DNA remained in the population at later times (14 days), the fact that H3K9me3 levels remained

high on the marker gene while dropping on alphoid^{tetO} DNA suggests that even after tTS binding, subtle differences in chromatin structure remain between the (now inactive) alphoid array and flanking regions.

Taken together, ChIP results demonstrate that tTS binding to the alphoid^{tetO} array increases the level of heterochromatic markers on the array, and disrupts CENP-A chromatin. Some of these changes were transitory, reflecting the possible flexible nature of some epigenetic states.

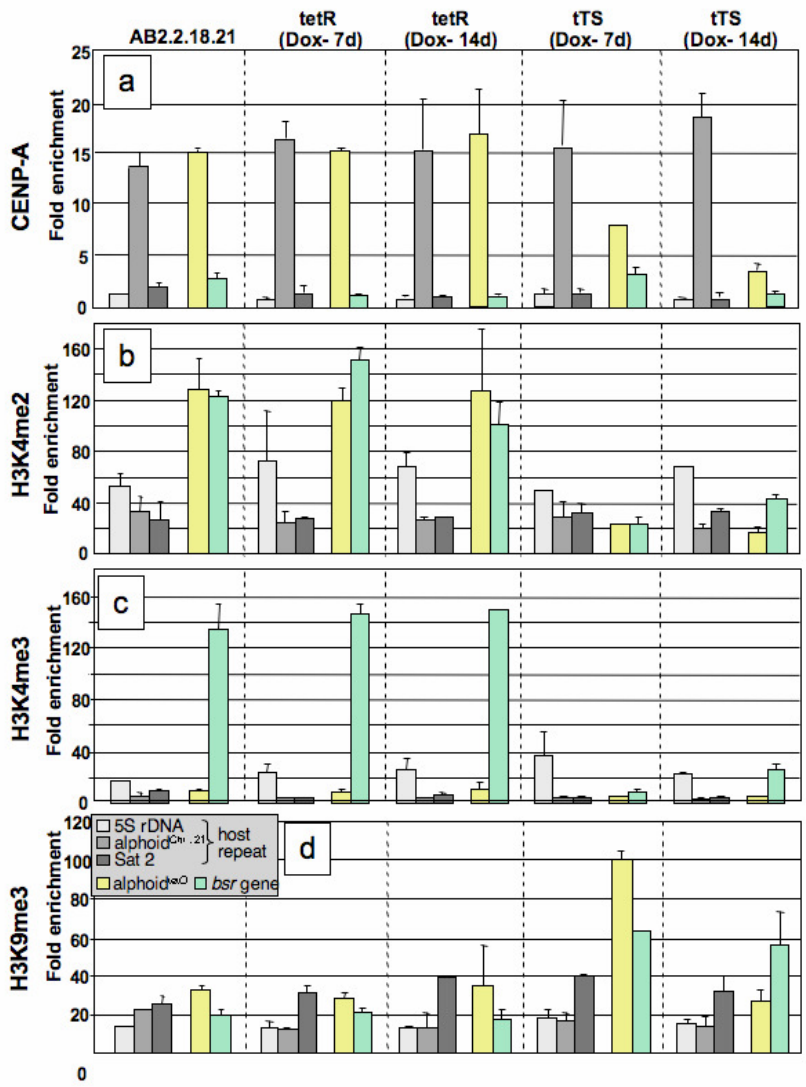


Figure 21. ChIP analysis of alphoid^{tetO} HAC chromatin

ChIP with antibodies for CENP-A, H3K4me2, H3K4me3 and H3K9me3 on the AB2.2.18.21 cell line. To quantify the precipitated DNA, qPCR with oligonucleotides specific for the alphoid^{tetO} array, the Bsr gene, ribosomal DNA (rDNA), alpha-satellite DNA (sat-2) and Chromosome 21 alphoid sequences (11-mer) was used. An average of 3 experiments is plotted (data of Megumi Nakano).

4.2 **tTS induces loss of kinetochore components**

The binding of tTS:YFP to the alphoid^{tetO} HAC caused major changes in the underlying chromatin, resulting in a loss of the alphoid^{tetO} HAC from the population of cycling cells. These data suggest that tTS caused a severe disruption of kinetochore functions, which led to mitotic instability.

To check if tTS targeting caused changes in the association of kinetochore components, I transfected AB2.2.18.21 cells with TetR:YFP and tTS:YFP, fixed the cells at 48 hours after transfection and stained for core kinetochore proteins. For the immunostainings I used antibodies for CENP-B and CENP-C, and also human ACA antiserum.

In a fraction of the stained nuclei analyzed in interphase, while CENP-C was strongly bound to the TetR-associated HACs (Figure 22a), the tTS-targeted HAC lost any detectable staining for CENP-C (Figure 22b).

The staining of transfected mitotic cells showed that HACs targeted with TetR:YFP were normally aligned on the metaphase plate, and stained positively for CENP-C (Figure 22c). However, the binding of tTS:YFP often caused the HAC to localize near the edge of the congressed chromosomes. These HACs did not assemble core kinetochore proteins, as they were negative for CENP-C, CENP-B and ACA antigens (Figure 22d-e).

Together these results indicate that the targeting of the tTS silencer, which disrupts CENP-A chromatin at the alphoid^{tetO} sequences (ChIP data, Figure 21), leads to the dissociation of inner kinetochore proteins like CENP-C. CENP-C is part of the CCAN complex, which is fundamental to establish a functional kinetochore. The disruption of the HAC kinetochore would be predicted to result in defective mitotic segregation.

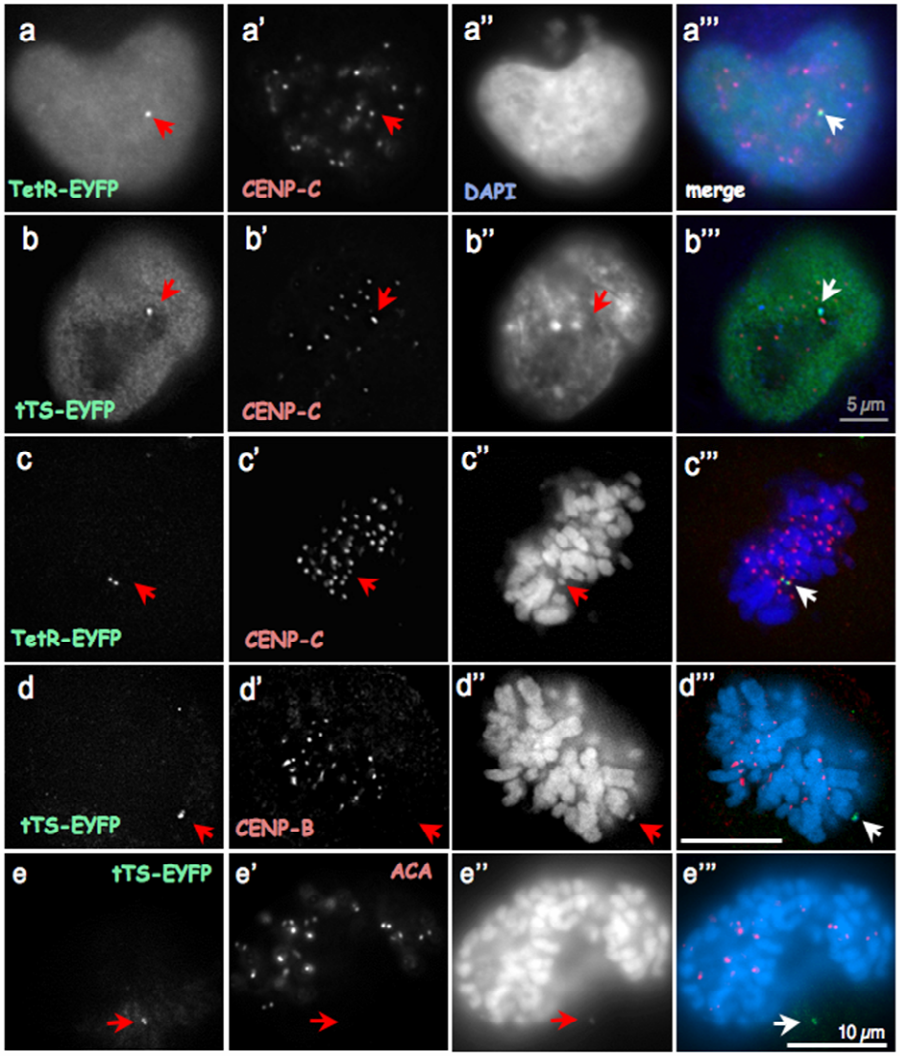


Figure 22. Disruption of the pre-kinetochore
Cells were transfected with TetR:YFP (a, c) and tTS:YFP (b, d, e) and fixed 48 days post-transfection for immunostaining with anti-CENP-C (a'-c'), anti-CENP-B (d') and ACA (e') antibodies.

4.3 HAC loss is caused by non-disjunction and formation of nano-nuclei

The cytological analysis of AB2.2.18.21 cells transfected with either tTA:YFP and tTS:YFP showed a progressive loss of the alphoid^{tetO} HAC from the cultured cells (Fig. 18-19). The fusion to TetR to both enzymes did not seem to affect their transcriptional activity on the targeted tetO array.

Karyotype abnormalities can be caused by two main mechanisms: chromosome non-disjunction and chromosome loss, which respectively give a chromosome segregation ratio of 2:0 and 1:0. Chromosome loss can occur because of mis-segregation and non-inclusion in a daughter nucleus, but can also be caused by problems in replication.

The analysis of the number of alphoid^{tetO} HAC copies per nucleus showed that the increased number of nuclei with no HACs was not balanced by a corresponding increase in nuclei with 2 HACs (Note, the cell-by-cell assay scores only HAC signals in the nucleus) (Figure 18b). Therefore, the HAC seemed to be undergoing chromosome loss.

To better understand this mechanism, I analyzed the mitotic behaviour of the alphoid^{tetO} HAC in cells transfected with TetR:YFP, tTA:YFP and tTS:YFP. The presence of YFP on all targeting constructs facilitated the task of spotting the alphoid^{tetO} HAC amongst the pool of endogenous chromosomes. However, to distinguish the HAC I also used a BAC-based FISH approach.

In cells transfected with tTA:YFP and tTS:YFP, the HAC appeared in mitosis as a pair of YFP- or BAC-signals, corresponding to the two HAC sister chromatids. However, one or both sister HACs were often found lagging compared to the rest of segregating chromosomes (Fig. 23a, d). 7 days after transfection with tTS:YFP, 30% of cells contained lagging HACs (Fig. 23e). These data revealed that the targeted HAC apparently replicated in S phase, but was not able to sustain normal mitotic segregation. The fact that the binding of TetR:YFP to the alphoid^{tetO} HAC does not affect its ability to be retained in cycling cells strongly suggests that the mere binding of the tetR protein to the DNA does not affect DNA replication.

A detailed examination of transfected cells revealed the presence of minute DAPI-stained structures in the cytoplasm (Fig. 23b-d). These resembled tiny versions of micronuclei typically observed when chromosomes fail to segregate correctly in mitosis, and I refer to them as nano-nuclei. FISH analysis with a BAC-probe showed that these nano-nuclei were positive for HAC sequences (Fig 23d). Furthermore, HAC-containing nano-nuclei were negative for ACA staining (Fig. 23c) or CENP-A (Fig. 23d). After 7 days of tTS expression, about 11% of interphase cells had nano-nuclei containing alphoid^{tetO} HAC sequences (Fig. 23d-e).

Although a thorough characterization of these nano-nuclei has not been performed, I suggest that its inclusion in these tiny cytoplasmic structures ultimately results in loss of the HAC from the cell.

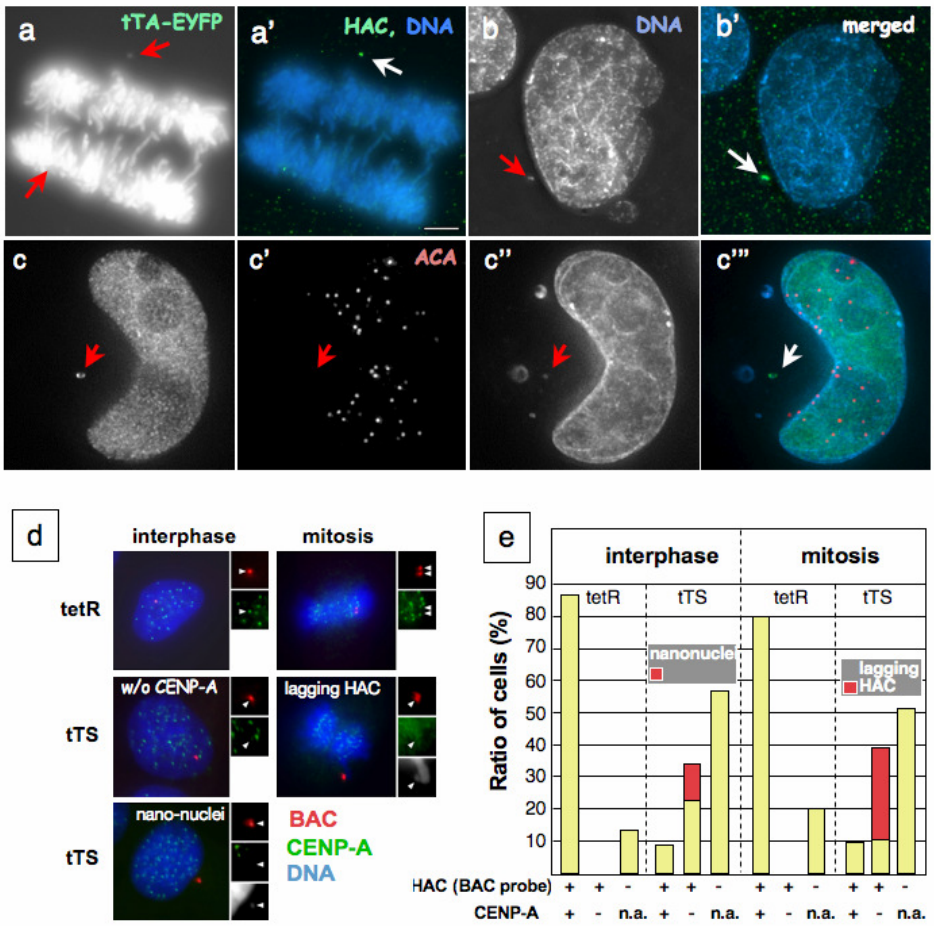


Figure 23. Mitotic HAC mis-segregation and inclusion in nanonuclei.
Alphoid^{tetO} HACs targeted with tTA:YFP (a) or tTS:YFP (b) failed to segregate with endogenous chromosomes, and are included into nanonuclei (b, c). The nanonuclei are negative for ACA staining (c'). **(d)** ImmunoFISH with a BAC-probe and anti-CENP-A antibody on cells transfected with TetR:YFP (top panels) or tTS:YFP (bottom panels). BAC-positive nanonuclei are negative for CENP-A staining. **(e)** Frequency of BAC-positive interphase nanonuclei or mitotic lagging HACs after transfection with TetR:YFP and tTS:YFP.
Data in (d) and (e) are from Megumi Nakano.

5. Conclusions

I have here described the construction and analysis of a new type of human artificial chromosome, containing arrays of sequences that can be manipulated *in vitro* or *in vivo* by targeting to it proteins of interest. For the formation in human fibrosarcoma HT1080 cells of stable artificial chromosomes, containing a de novo originated centromere element, transfected plasmids must contain an array of at least 50 kb of α-satellite monomers carrying the CENP-B-binding region (CENP-B box) (Ohzeki et al., 2002; Okamoto et al., 2007). We have designed an array of alphoid DNA where, together with CENP-B-binding sequences, tetOperator sequences were included. This ~350 bp dimer was expanded to a sequece of ~3.5 Kb through conventional cloning, then to an array of ~ 10 KbA strategy was designed by Vladimir Larionov to amplify the 3.5 kb array by rolling circle amplification, in the laboratory of Vladimir Larionov (NIH Bethesda-MA, USA). Transformation-Associated Recombination (TAR) cloning in yeast was used to expand the array to ~50kb (performed by Vladimir Noskov). TAR cloning is a technology that recently is being adopted for cloning large genomic regions, not only from higher vertebrates (humans i.e.) but also from prokaryotes.

This synthetic alphoid^{tetO} array was used in a HAC-formation assay (Ikeno et al., 1998) in the laboratory of Vladimir Larionov to obtain HT1080 cell lines carrying 1 copy per cell of the alphoid^{tetO}. This new type of alphoid^{tetO} HAC was stably retained by doubling cells and contained a type of alphoid chromatin overall similar to endogenous centromeric alphoid regions. Surprisingly, the tetO array embedded within the HAC was accessible to TetR-linked targeting proteins *in vivo*. The targeting of YFP- or RFP-fused TetR proteins bound to the alphoid^{tetO} HAC allowing the direct visualization of the HAC in all stages of the cell cycle. In addition, the binding of YFP/RFP-TetR proteins did not affect the replication or the mitotic behaviour of the alphoid^{tetO} HAC.

The targeting of a transcriptional activator (tTA) and a transcriptional repressor (tTS) both inactivated the HAC synthetic centromere. However, while tTA had a mild, cell-specific effect, tTS widely inactivated the alphoid^{tetO} DNA. In fact, tTS caused a change of the alphoid chromatin towards a more heterochromatic state and inhibited the basal (low) transcriptional activity of the alphoid^{tetO} DNA. tTS targeting caused inner kinetochore proteins to delocalize from the synthetic alphoid^{tetO} HAC. The HAC without a functional kinetochore structure was unable to properly align with endogenous chromosomes on the mitotic metaphase plate and properly segregate in anaphase. The destabilized HAC was ultimately included into tiny cytoplasmatic DAPI-positive structures (nano-nuclei) and presumably lost from the doubling cell.

The striking difference between tTA and tTS targeting to the alphoid^{tetO} centromere suggests that a balance between euchromatin and heterochromatin at centromeres is crucial for their function. While centromeres can sustain a certain degree of chromatin “openness”, heterocromatin dramatically kills centromere function. Alternatively, some specific chromatin types might not be compatible with centromere functionality. Also, a basal level of transcriptional activity might be necessary to preserve centromeric function and inheritance.

IV. Mechanisms of kinetochore inactivation

1. Introduction

The experiments described in Chapter III used a Human Artificial Chromosome (HAC) that was originally made in an HT1080 cell line. The most important achievements of the study were: 1) the production of a stable HAC containing completely synthetic sequences; 2) the creation of a HAC whose centromere function can be manipulated *in vivo*; and 3) the first direct evidence in human cells that an enrichment of heterochromatin markers is not compatible with centromere functionality.

Unfortunately, the HT1080 cell line has several disadvantages for a wider application of the tetO-containing alphoid HAC. In particular, since we had demonstrated the possibility of targeting specific proteins to the Artificial Chromosome *in vivo,* we considered targeting specific constructs *in vitro*. Purification of the alphoid^{TetO} HAC from cultured cells via a tagged-TetR anchor would allow a mass spectrometry analysis of the protein composition of the stable artificial chromosome. This would be of great interest, because the HAC essentially contains only kinetochore chromatin. However, one of the key factors of a successful proteomic analysis is the amount of starting material, which depends on the number of cells. The isolation of a human artificial chromosome from the pool of endogenous chromosomes is difficult to achieve. In fact, the HAC contains only ~2 Mb of DNA and it is much smaller than normal human chromosomes.

Human fibrosarcoma HT1080 cells grow adherent to a surface with a doubling time of about 30 hours. Furthermore, using conventional lipid-based transfection reagents, transfection efficiencies were highly variable, ranging from 50% to 80%. This limitation, combined with the impossibility to obtain a large number of cells, made the cell line unsuitable for a large-scale proteomic study.

HeLa cell lines are available that can be grown in suspension in stirred media, allowing a much higher number of cells to be obtained, compared to monolayer cultures. In our laboratory we can achieve transfection efficiencies

with HeLa cells of up to 90%. This prompted us to attempt the transfer and characterization of the alphoid^{TetO} HAC in our fast-growing HeLa cells. To date, there is no published study of how an artificial chromosome behaves in HeLa cells.

2 Construction and analysis of a HeLa cell line containing alphoid^{TetO} HAC

A HeLa cell line stably expressing resistance to the drug Geneticin was used for Poly-ethyleneGlycol (PEG)-mediated cell fusion with our AB2.2.18 line, which contains the alphoid^{tetO}HAC with its Blasticidin resistance gene. The hybrid HT1080-HeLa clones were isolated by co-selecting for Geneticin and Blasticidin resistance. Fluorescence *In Situ* Hybridization (FISH), with a BAC-based probe, was then used on chromosome spreads to select cell lines containing only one copy of the HAC per cell (Figure 24a).

The cell line, 1C7, was selected because ~ 90 % of cells carried 1 copy of the alphoid^{TetO} HAC per cell, as determined by FISH. The number of chromosomes per nucleus was much higher than a normal diploid nucleus, as expected for a hybrid cell line, and the doubling time was ~ 20 hours, similar to HeLa cells (Figure 24a-b).

To analyse the mitotic stability of the alphoid^{TetO} HAC, 1C7 cells were cultured in the absence of Blasticidin, but in presence of 500 ng/µl of Geneticin. 1C7 cells were left to attach onto poly-Lysine slides and fixed with Carnoy's fixative for FISH analysis with a BAC probe. Samples before and after selective growth were prepared. After 49 generations in culture with no drug selection, about 80% of the cells retained the HAC in the nucleus, mainly as 1 copy per cell (Figure 24d). In comparison, cells that were grown with drug selection for the same number of generations showed ~87% of nuclei containing one or two copies of the alphoid^{TetO} HAC (Figure 24d). This

result demonstrated that the HAC was mitotically stable and retained by the hybrid 1C7 cells during several cell divisions.

To test the association of kinetochore proteins to the alphoid^{tetO} HAC, I used immunostaining coupled to FISH (ImmunoFISH) on chromosome spreads prepared from 1C7 cells. The staining for CENP-C showed that this inner kinetochore component localized to the HAC as a double spot in mitosis (Figure 24c). This suggests that the alphoid^{tetO} HAC assembles a proper kinetochore structure and undergoes a normal replication yielding paired sister chromatids.

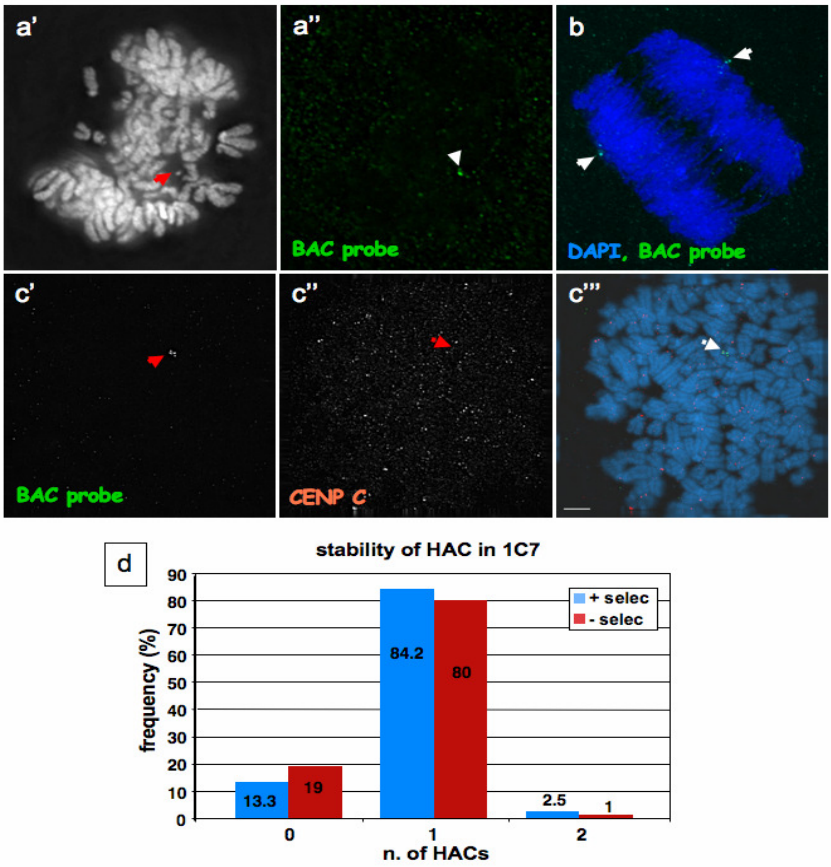


Figure 24. Analysis of alphoid^{tetO} HAC in 1C7 HeLa cell line.

(a-b) FISH with a BAC-probe (a'', b) on the 1C7 cell line containing the Human Artificial Chromosome in prometaphase (a) or anaphase (b). **(C)** ImmunoFISH with a BAC-probe (c') and an antibody against CENP-C (c''). These images show that the HAC binds the kinetochore protein CENP-C. **(d)** analysis of stability of the alphoid^{tetO} HAC in 1C7 cells cultured with (+ selec, blue) or without (- selec, red) Blasticidin selection. The graph shows the frequency (%) of nuclei containing 0, 1 or 2 HACs, after 49 generations.In all micrographs arrows point to the HAC.

3 Destabilization of the alphoid^{tetO} HAC by KAP1

3.1 Introduction

The role of centromeric DNA in the assembly of centromere chromatin components is still unclear. Only alphoid DNA and CENP-B boxes, but not other sequences from human chromosomes, are required for de novo centromere assembly (Ohzeki et al., 2002; Saffery et al., 2001; Harrington et al., 1997). On the other hand, centromeric sequences are highly divergent and other than the presence of CENP-B boxes, no sequence conservation is observed between human alphoid DNA and mouse centromeric minor satellite DNA. Furthermore, CENP-B and the CENP-B box, which are important for *de novo* centromere formation (Ohzeki et al., 2002), have not been detected in the alphoid DNA on the Y chromosome (Haaf et al., 1995) or in neocentromere-specific DNA sequences (Saffery et al., 2001). These active centromeres, yet lacking of DNA-bound CENP-B, raise the question how centromeric inheritance is maintained without *de novo* assembly mechanisms (Tyler-Smith et al., 1999).

In HT1080 cells, constructs linked to the protein TetR can specifically bind to an array of TetO sequences embedded in the alphoid DNA of a Human Artificial Chromosome (HAC). The presence of YFP in the targeting constructs allows the visualization of the HAC in transfected nuclei and the direct analysis of protein composition by immunostaining. In this cell line, the targeting of a transcriptional activator (tTA) and, more strikingly, of a transcriptional repressor (tTS), impaired the centromeric functionality of the alphoid^{tetO} HAC. In conclusion, these experiments showed that an increase of heterochromatin-like epigenetic modifications is not compatible with the recruitment of a functional kinetochore structure (e.g. Chapter III).

To analyze the downstream players in the loss of centromeric function following targeting of tTS:YFP, I studied the principal mediator of tTS activity: KAP1. The Kruppel-associated box (KRAB) domain of tTS was derived from

the human kidney protein Kid-1 and is a KRAB domain typical of KRAB-zinc finger transcription repression proteins. It is still not clear mechanistically how KRAB domains repress transcription, but repression seems correlated with the binding to KAP1 (KRAB associated protein 1), a transcriptional regulator also known as TIF1β (Friedman et al., 1996). Several experimental results support the hypothesis that KAP1 is a scaffold protein with a key role as an intermediate for the repression of transcription by the KRAB-Zn^{2+} Finger proteins: (i) KAP1 binds multiple KRAB repression domains both *in vivo* and *in vitro*; (ii) KRAB domain mutations that abolish repression decrease or eliminate interaction with KAP1; (iii) KAP1 directly tethered to DNA is sufficient to repress transcription; and (iv) a KRAB domain does not exhibit repression in cells which lack KAP1 protein. (Agata et al., 1999; Kim et al., 1996; Le Douarin et al., 1996; Moosmann et al., 1996; Nielsen et al., 1999; Schultz et al., 2001)

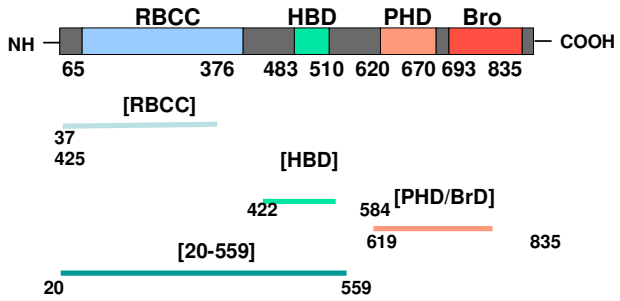
KAP1 reveals several well-conserved consensus signature motifs. Starting from the N-terminus, KAP1 contains a RING finger motif followed by two B-boxes, spanning from residues 61 to 260, a coiled-coil region from 260 to 410, and a region binding heterochromatin protein I (HPI) from 483-510. At the C-terminus, the protein contains two domains which are of interest because of their involvement in chromatin remodelling mechanisms: a PHD finger and a bromodomain (from 625 to 835) (Friedman et al., 1996).

The RBCC domain (RING finger – B boxes – coiled coil) situated at the N-terminus of KAP1 is a protein-protein binding surface. The RING finger and B-boxes have been shown to be required for binding the KRAB domain, since an amino-terminal deletion (KAP1[239-835]), that includes the coiled-coil region and the reminder of the protein, is not able to bind to GST-KRAB (Friedman et al., 1996). Detailed analysis showed that binding to the KRAB domain requires structural elements of all sub-motifs. KAP1-RBCC is also involved in homo-oligomerization, which is necessary but not sufficient for KRAB binding, implying that distinct functional regions may be present for oligomerization and KRAB domain binding (Peng et al., 2000). The KAP1-

binding does not affect the ability of KRAB-Zn Finger proteins to bind the DNA.

The HPI-Binding domain (HBD) was identified by sequence comparison between KAP1 and hTIF1α, which detected a region of KAP1 (residues 483-510) of high homology to a sequence of hTIF1α (residues 672 to 698) involved in the direct binding to HPIα. Further characterization showed that indeed KAP1 directly interacts with the HPIα chromoshadow domain via a core PxVxL motif *in vitro* and *in vivo* (Lechner et al., 2000). In addition, disruption of the binding to HPIα by mutation of the HPI-binding motif decreases the transcriptional repression activity of KAP1 (Sripathy et al, 2006).

At the C-terminus, KAP1 contains some very interesting features. The PHD finger is structurally similar to RING domain, binds to atoms of Zinc and is implicated in protein-protein interactions (Capili et al., 2001; reviewed in Bienz M, 2006), whereas the bromodomain specifically bind acetylated histone tails. These two domains together were able to significantly repress transcription when individually tethered to the DNA upstream of a reporter gene. A wild type level of repression was reconstituted only when the two domains were targeted together (Schultz et al., 2001). This result suggests that the two domains function cooperatively and represent a functional unit of KAP1 independent from HPI-binding. These domains seem to repress transcription by recruiting the histone deacetylase complex NuRD and the H3K9-specific methyltransferase SETDB1 (Schultz et al., 2001; 2002). These data imply an additional repression activity associated with KAP1 that does not involve the recruitment of HPI proteins.



3.2 Targeting of tTS:YFP

To assess if transcriptional repressors could inactivate a stable HAC regardless of the host cell line, HeLa 1C7 cells were used for the same type of approach applied in HT1080 cells (see Chapter III). Cells were transfected with tTS:YFP or TetR:YFP constructs and stained with human ACA autoantibody 48 and 72 hours after transfection. ACA autoimmune sera recognizes several kinetochore proteins with a preference for CENP-B (Earnshaw and Rothfield, 1985). 48 hours after transfection with tTS:YFP, the amount of ACA staining associated with the HAC decreased to 60% compared to control TetR:YFP (>130%) (values are calculated in respect the average signal at endogenous centromeres) (Figure 25a).

To further test if targeting of tTS:YFP impaired the mitotic stability of the HAC kinetochore, I transfected 1C7 cells with TetR:YFP and tTS:YFP. Transfectants were then selected with 1.5 μg/ ml of Puromycin 24 hours after transfection. At 7 days post-transfection, cells were fixed with Carnoy's fixative and processed for FISH analysis. Counting the number of HAC signals in interphase nuclei showed a marked loss of the artificial chromosome in the population transfected with tTS:YFP compared to TetR:YFP (not shown). In cells transfected with tTS:YFP, micrographs taken at low magnification showed many nuclei with no FISH signal (Figure 25c, yellow arrows), interspersed with nuclei showing a bright signal for the HAC (Figure 25c, red arrows). In cells transfected with TetR:YFP, almost all nuclei observed retained the HAC (Figure 25b, red arrows).

Thus far, this study shows that also in a HeLa-derived cell-line, tTS:YFP is able to disrupt the centromeric activity of the alphoid^{tetO} HAC.

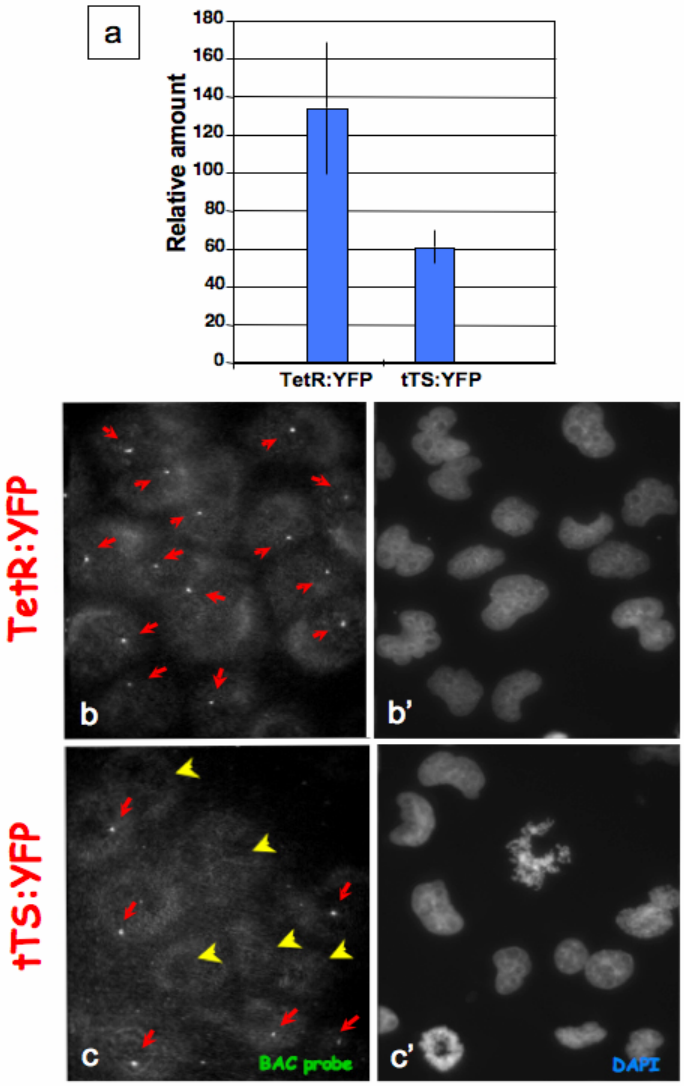


Figure 25. tTS:YFP targeting to the alphoid^{tetO} HAC

(a) Quantification of ACA staining of the alphoid^{tetO} HAC targeted with TetR:YFP and tTS:YFP.The amount of signal colocalizing with the YFP (HAC signal) was compared to the average ACA staining of endogenous centromeres (relative amount, %). **(b-c)** FISH with a BAC probe (B, C) on cells transfected with the indicated constructs and cultured for 12 days. (B′, C′) DAPI staining. Yellow arrows indicate nuclei with no HACs.

3.3 Construction of TetR:YFP fusions of full-length KAP1 and its domains

Wild type KAP1 ORF, obtained from cDNA and coding amino-acids 20-835 (KAP1) was generously provided by Dr. David Schultz (The Wistar Institute, Philadelphia-PA, USA). This construct, lacking the first 20 residues, demonstrated transcriptional repression activity similar to full-length KAP1 when used as GAL4-fusion on a reporter gene (Sripathy et al., 2006). As KAP1[20-835] has similar repression activity compared to the full-length protein, I will refer to it as KAP1Δ19. To dissect the role of the different domains of KAP1 to render the HAC non-functional, different truncations of the protein were constructed. To identify which, if any, functional region of KAP1 might be responsible for HAC kinetochore inactivation, the following constructs were used for targeting analysis: KAP1[HBD], coding for the HPI-binding region between residues 422 and 584; KAP1[PHD/BrD], coding the PHD finger and bromodomain of the protein localized from residue 619 to 835; KAP1[20-559], coding for the N-terminus up to residue 559 and lacking the PHD finger bromodomain; KAP1[37-425], coding for the RBCC motifs (all oligonucleotides used for PCR amplification are listed in Materials and Methods). The full-length protein and all truncated constructs were linked to the C-terminus of TetR:YFP in the modified pEYFP-C1 vector backbone.

3.4 Dissociation of CENP-A, CENP-C and CENP-H from the alphoid^{tetO} HAC

To analyze the effect of the binding of KAP1 on the assembly of pre-kinetochore components, I transfected HeLa-1C7 cells with TetR:YFP: KAP1Δ19, truncation mutants and control TetR:YFP. Similarly to the expression of tTS:YFP, and localization observed in human HT1080 cells, all constructs fused to TetR:YFP localized predominantly in the nucleus, where

one or rarely two bright spots could be observed. The presence of YFP in the targeting constructs allowed the visualization of the HAC in transfected cells. Only cells expressing at similar levels were selected for further analysis.

The first inner kinetochore protein analyzed was CENP-H. The analysis of the association of CENP-H to the targeted alphoid^{tetO} HAC revealed that this proteins was easily delocalized by KAP1Δ19 and its single domains, while CENP-A remained associated to the HAC chromatin. In fact, between 33 and 47% of the nuclei showed CENP-A (+)/CENP-H (-) staining on the targeted HAC (Figure 26b) with all KAP1 constructs but not with control TetR:YFP. Importantly, a difference could be observed between the full-length construct and its sub-domains on the association of CENP-A with the synthetic HAC. Only KAP1Δ19, but not the subcloned single domains, caused simultaneous delocalization of CENP-A and CENP-H in 47% of cells (Figure 26c). This suggests that the effect of the almost-full-length protein is more severe than the truncated constructs. In cells transfected with TetR:YFP, all nuclei analyzed showed binding of both CENP-proteins to the HAC (Figure 26a).

To further dissect the effect on the mutual localization of other pre-kinetochore components and CENP-A, I transfected cells with KAP1Δ19 and the truncated constructs, fixed the cells 48 hours post-transfection, and co-stained with antibodies for CENP-A and CENP-C. In cells expressing control TetR:YFP, 90-95% of HACs co-localized with CENP-A and CENP-C, confirming that the targeting of TetR:YFP does not affect pre-kinetochore assembly (Figure 27a), similarly to that observed for HT1080 cells (e.g. Chapter III). In contrast, the targeting of KAP1Δ19 had a strong effect on the centromeric function of the alphoid^{tetO} HAC. In 58% of the nuclei analyzed, CENP-C was not associated with the HAC and no residual staining could be observed (Figure 27b). Interestingly, in 50% of the nuclei where CENP-C association to the alphoid^{tetO} DNA was disrupted, CENP-A was still associated with the kinetochore (Figure 27b). In the remaining 50% of HACs where the CENP-C staining was lost, CENP-A was also lost from the alphoid^{tetO} DNA (not shown). Surprisingly, the targeting of just the HPI-

binding domain (KAP1[HBD]) and the PHD/Bromodomain (KAP1[PH/BrD]) had a weak effect in delocalizing CENP-C from the HAC kinetochore (6.8% and 12.5% respectively, Figure 27e-g). Unexpectedly, the targeting of just the RBCC region (NH2-terminus) of KAP1 was able to strongly affect CENP-C localization (40%, Figure 27d, g) and this effect showed a 2-fold increase when the HPI-binding region was added to the N-terminal RBCC motifs (KAP1[20-559], Figure 27c, g). In most of transfected cells, CENP-C was completely lost from the targeted HAC and could not be detected by immunostaining. Furthermore, in a fraction of transfected cells, CENP-A was still associated with the alphoid^{tetO} DNA in the absence of CENP-C (Figure 27d).

In conclusion, KAP1 is able to disrupt the association of pre-kinetochore components while retaining CENP-A chromatin. CENP-H appears to dissociate more readily than CENP-C. Therefore, and surprisingly, the disruption of CENP-C binding is not a consequence of the loss of CENP-A nucleosomes from the targeted alphoid^{tetO} array.

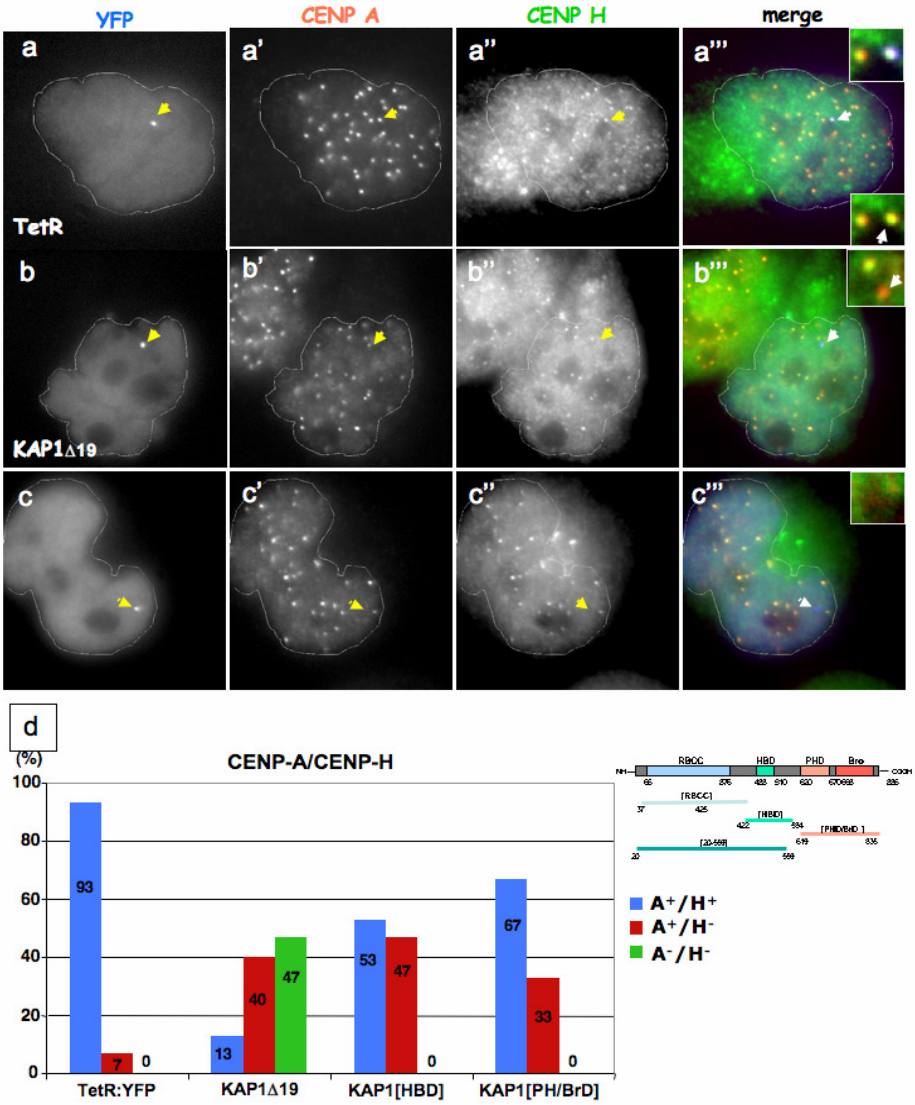


Figure 26. Immunostaining for CENP-A and CENP-H.

Cells were transfected with the indicated TetR:YFP fusion constructs (a-c) and selected with puromicin 24 hours after transfection. At 96 hours cells were fixed and stained with antibodies against CENP-A (a'- c') and CENP-H (a''- c''). Arrows indicate the HAC. **(D)** quantification of the number of HACs positive (+) or negative (-) for CENP-A or CENP-H.

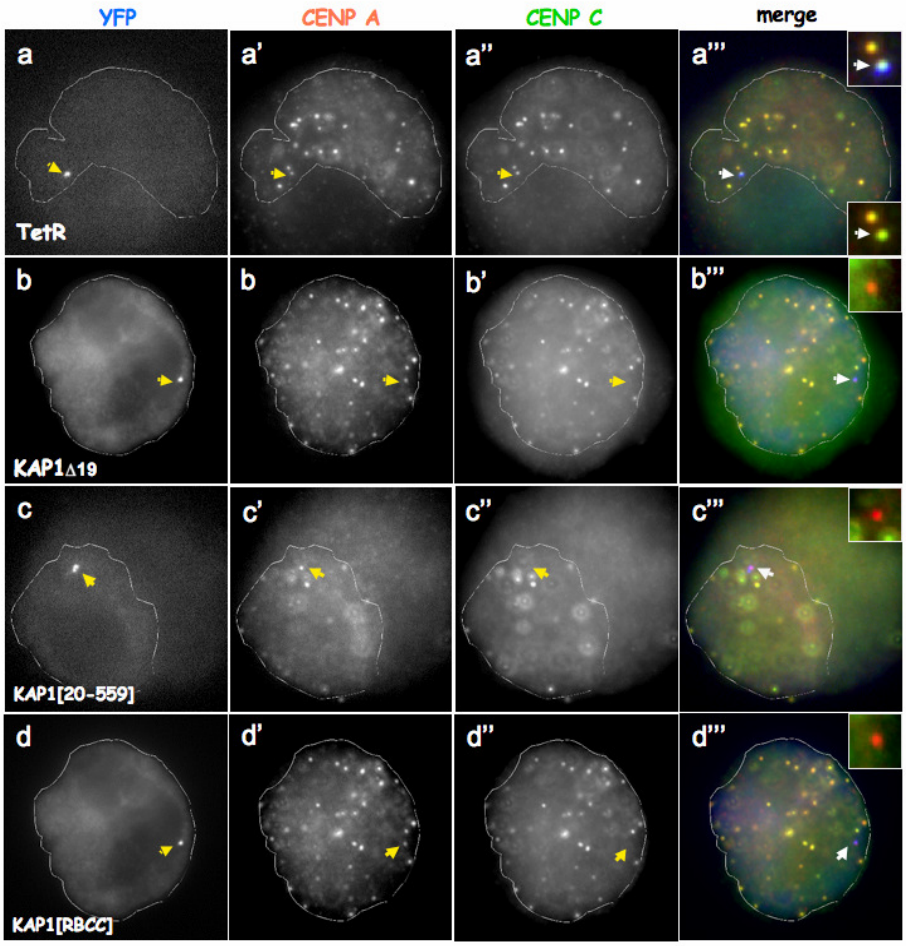

YFP
CENP A
CENP C
merge
a
a'
a''
a'''
TetR
b
b
b'
b'''
KAP1Δ19
c
c'
c''
c'''
KAP1[20-559]
d
d'
d''
d'''
KAP1[RBCC]

Figure 27

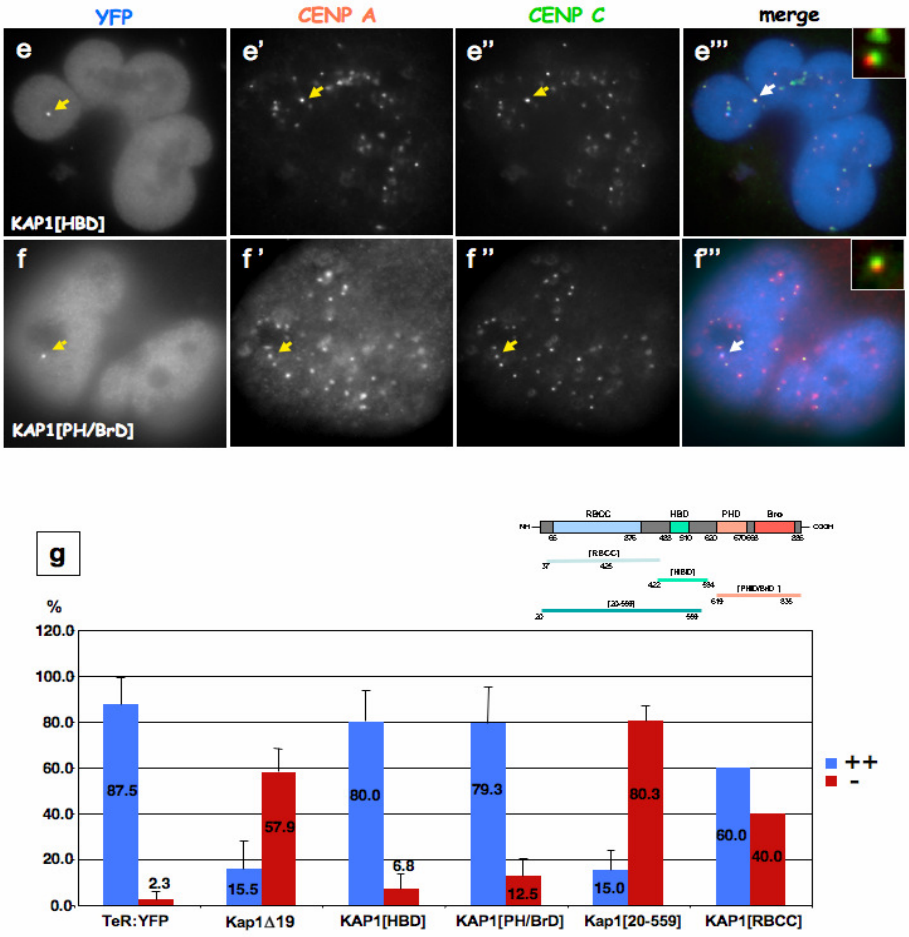


Figure 27. Association of CENP-A and CENP-C.

(a-f)1C7 cells were transfected with the indicated constructs (a-f) and fixed at 96 hrs for staining with anti-CENP-A (a'-f') and anti-CENP-C (a''-f'') antibodies. Arrows point to the HAC. **(g)** quantification of the CENP-C associated (+) or not (-) to the targeted HAC.

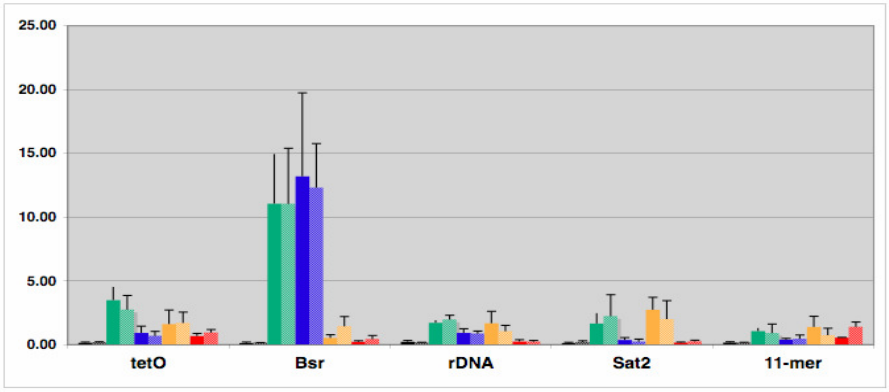
3.5 Chromatin Immunoprecipitation analysis of the HAC chromatin

The immunostaining analysis revealed that targeting KAP1Δ19 to the alphoid^{tetO} HAC impaired the ability of the alphoid^{tetO} array to bind kinetochore components. In addition, co-staining for CENP-A and CENP-C suggested that the dissociation of CENP-A might be secondary to the disruption of pre-kinetochore assembly. To test whether, following the targeting of KAP1Δ19, the HAC chromatin was altered; I used chromatin immunoprecipitation (ChIP) to precipitate DNA associated with specific types of modified histones, and qPCR to quantify the amount.

1C7 cells were transfected with control vector, carrying only the gene for Puromicin resistance (puro), or with TetR:YFP: KAP1Δ19, and cultured for 4 days before fixation. For the ChIP, antibodies against H3K4me2, H3K4me3, H3K9me3 and CENP-A were incubated with cell lysates. To measure the amount of precipitated HAC sequences, I used qPCR with oligonucleotides that amplify the HAC tetO sequence, or a region of the gene coding for the resistance to Blasticidin (Bsr). As control, I used oligonucleotides that amplify endogenous sequences usually enriched for specific histone modifications. To normalize the H3K9me3 histone modification signal, which is a marker for heterochromatin, I used endogenous α-Satellite DNA (sat-2). Actively transcribed ribosomal DNA sequences (rDNA) were used as a control for H3K4me2 and H3K4me3 modifications. For CENP-A, I used oligonucleotides specific for the alphoid consensus sequence of Chromosome 21 (11-mer).

The comparison between puro- and KAP1Δ19-transfected samples indicated that KAP1 targeting caused a decrease of either H3K4me2 and H3K4me3 modifications associated to HAC tetO and Bsr sequences (Figure 28). This could be observed on either raw non-normalized (Figure 28a) or control-normalized values (Figure 28b). In contrast, H3K9me3 was increased on both tetO and Bsr sequences. CENP-A associated with the alphoid^{tetO} DNA appeared diminished to ~50% compared to control only after normalization with control 11-mer sequences.

ChIP analysis showed that in a time as short as 4 days post-transfection, it is possible to observe a change in the histone tail modifications associated to the HAC alphoid^{tetO} and Bsr sequences. Consistent with the immunofluorescence analysis, a drop of ~50% of CENP-A associated to the HAC synthetic alphoid could also be detected.



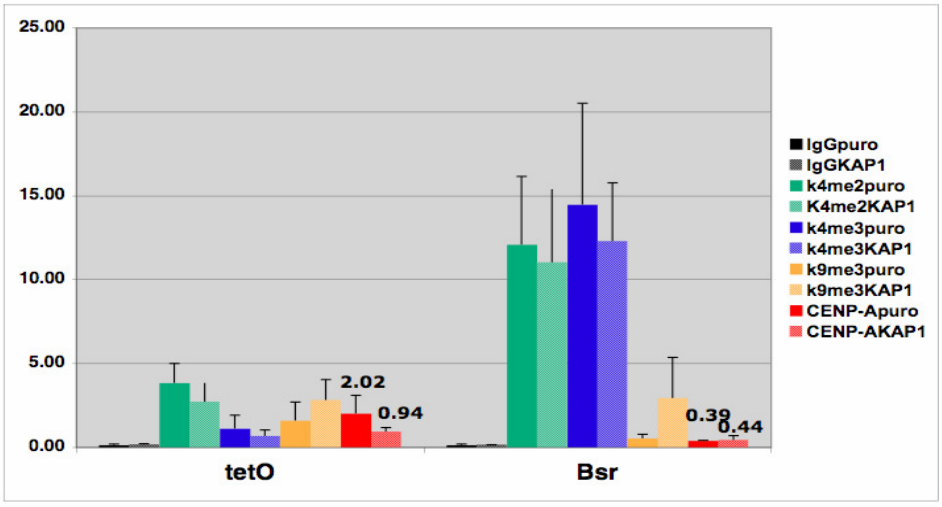


Figure 28. ChIP of the HAC sequences

Cells were transfected with control vector (Puro, full colors) and TetR:YFP:KAP1 (shaded colors). After 4 days of culture, cells were fixed for ChIP analysis with the indicated antibodies. The amount of Immunoprecipated HAC DNA was quantified by qPCR using oligonucleotides specific for the tetO sequence and the blasticidin gene (Bsr). As internal PCR control, oligonucleotides for endogenous ribosomal DNA, Chromosome 21 alphoid DNA and aSatellite DNA were used in parallel PCR reactions. **(b)** qPCR with oligonucleotides for tetO and Bsr gene, normalized for the endogenous DNA sequences precipitated with the indicated antibodies.

3.6 The targeting of KAP1Δ19 to the synthetic HAC disrupts the association of Ndc80/HEC1

After analyzing the association of pre-kinetochore components to the targeted alphoid^{tetO} DNA, I looked at the recruitment of an important structural outer kinetochore protein, HEC1. HEC1 (or hNdc80) is part of an important kinetochore complex of 4 different subunits, which is involved in the regulated attachment of the kinetochore to the mitotic microtubules (MT). HEC1 localizes at the outer kinetochore plate from late G2 phase until the end of mitosis.

To determine the extent of kinetochore disruption, the association of HEC1 with the kinetochore was compared to that of inner proteins CENP-A and CENP-C. In cells transfected with TetR:YFP, the alphoid^{tetO} HAC aligned in the middle of the metaphase plate with the two YFP-positive parts highly stretched across the aligned endogenous chromosomes (Figure 29a). However, the YFP pair of dots on the HAC targeted with TetR:YFP: KAP1Δ19 appeared often not to be stretched (Figure 29b-d, f-g), and only one CENP-C signal was observed and colocalized with HEC1 in the middle part of the two unresolved YFP signals (Figure 29b-c).

This result suggested a lack of centromere tension, and this led us to analyze the localization of the outer kinetochore protein HEC1 upon targeting. Indeed, the localization of HEC1 was abnormal. In 30% of the nuclei analyzed, pairs of YFP dots that were positive for CENP-A or CENP-C staining (HAC) were negative for HEC1 (Figure 29b-d, f-h). This was particularly evident on targeted HACs that appeared as unresolved and unaligned with the congressing chromosomes (Figure 29d, g). In addition, HACs that had lost staining for CENP-A, or CENP-C, and HEC1 could also be observed (not shown).

Based on this analysis, I conclude that the targeting of KAP1Δ19 to the alphoid^{tetO} impairs the assembly of two functional kinetochore substructures necessary to sustain spindle MT attachments.

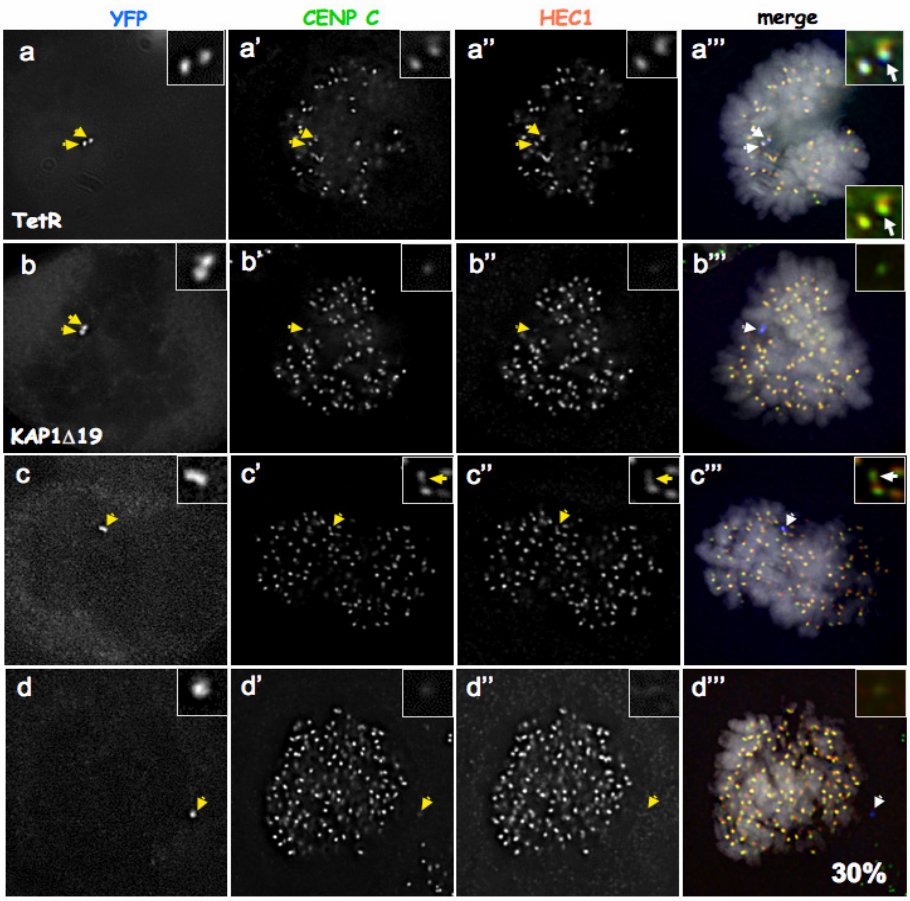


Figure 29.

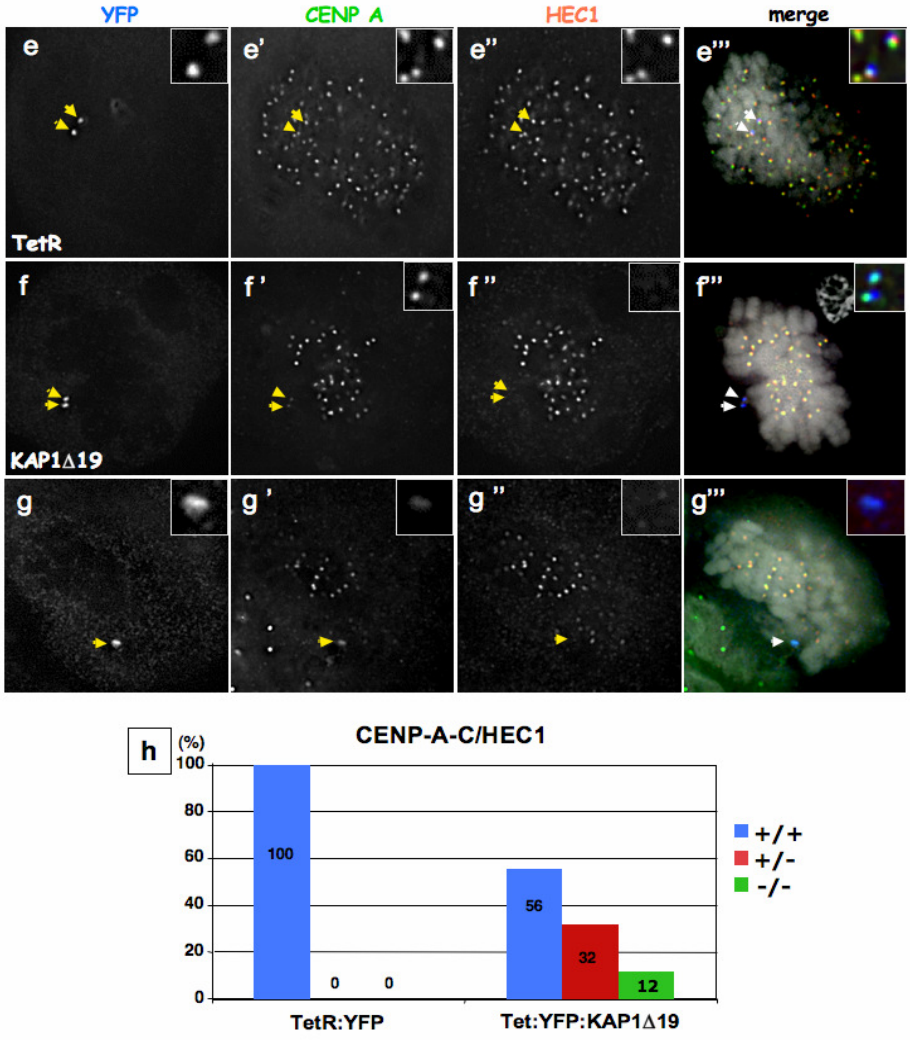


Figure 29. Co-localization of CENP-A, CENP-C and HEC1.
Cells were transfected with the TetR:YFP (a, e) or TetR:YFP:KAP1D19 (b-d, f-g) and stained with antibodies against CENP-A/C (e'-g'/a'-e') and HEC1 (a''-g'') 96 hours post-transfection. Arrowheads point to the HAC. **(I)** quantification of the number of cells positive (+) or negative (-) for CENP-A/C or HEC1.

3.7 Analysis of retention of alphoid^{tetO} HAC in transfected cells

Chromosomes without a functional kinetochore structure are not able to congress to the metaphase plate and fail to segregate normally in anaphase. The lagging chromosome is often included in neither of the two daughter nuclei, and is ultimately lost from the cell. A higher loss rate of chromosomes in cycling cells can be a measure of the lack of proper mitotic function of cells, but it can also be used to measure a lack of stability of single chromosomes. Hence, I studied the mitotic stability of the alphoid^{tetO} HAC, upon targeting with KAP1 and its different sub-domains.

In one approach, cells were transfected with the different targeting constructs and selected with puromycin 24 hrs post-transfection. Stable transfectants were grown under selection for up to 30 days. Samples of the cells were collected after 9, 18 and 30 days and genomic DNA was prepared for q PCR. The copy-number of the alphoid^{tetO} HAC was quantified by qPCR using oligonucleotides that specifically anneal to the TetO sequence and the Blasticidin resistance gene (Bsr). The amount of HAC-specific sequences obtained by RT-PCR was normalized for the actual amount of starting template by amplifying endogenous DNA sequences. In particular, for the normalization, oligonucleotides that amplify endogenous ribosomal DNA (rDNA) and Chromosome 21 alphoid DNA (11-mer) were used.

Since it was possible to observe complete loss of some kinetochore core proteins already within 48 hours after transfection (see figures 26-27), the effect on HAC copy-number was quantified after 9 days post-transfection. Indeed, different targeting constructs showed marked different effect on the alphoid^{tetO} HAC copy-number. For example, KAP1Δ19 caused a ~60% decrease of HAC-specific sequences (used as a measure for HAC retention), which is expressed as a normalized qPCR value for the starting template of 0.37 (1.01 is the control value for TetR:YFP). The truncated KAP1[20-559] (containing the NH2-terminus and the HPI-binding domain) also showed a marked destabilization of the HAC, yielding a value of 0.52 (~50% loss). The HPI-binding domain and PHD/Bromodomain - KAP1[HBD] and KAP1[PH/BrD] - had no significant effect on HAC stability compared to

control. Remarkably, transfection of the N-terminus KRAB-binding domain without the HPI-binding sequence (KAP1[RBCC]) was also able to perturb the HAC's segregation in mitosis, showing a drop of ~65% of the HAC template in the population of transfected cells (value 0.53) (Figure 30a).

A second cytological approach was also used to test the mitotic stability of the alphoid^{tetO} HAC in transfected cells. I used an experimental strategy similar to the one used for the analysis of HAC stability in the HT1080 (AB2.2.18.21) cell line (see Chapter III). Cells were transfected and selected with 3 μg/ml of Puromycin for 24 hours before fresh medium was added to the cells. The selection was performed 1 and 5 days after transfection. Following incubation for 12 days, cells were trypsinised, allowed to pre-attach onto Poly-Lysine-coated slides and fixed for Fluorescence In-Situ Hybridization (FISH) to count the number of alphoid^{tetO} HACs retained in interphase nuclei. Since the resistance gene for puromycin is on the same vector carrying the targeting construct, for the following data analysis I assumed that all cells resistant to the drug also express the targeting construct.

In the population of cells transfected with control TetR:YFP, approximately 20% of the nuclei had no HAC signal, whereas for the cells transfected with KAP1Δ19 and KAP1[20-559], the proportion of nuclei with no signal increased to >60%. The targeting of either the HPI-binding domain alone KAP1[HBD] and of the other two NH2-terminal domains KAP1[PH/BrD] had either mild or no significant effect on the alphoid^{tetO} HAC stability compared to control (Figure 30b).

The analysis of HAC retention in cultured 1C7 cells demonstrated that the association of KAP1Δ19 with alphoid^{tetO} DNA sequences of the synthetic HAC impaired its ability to segregate normally during mitosis. This was probably due to a loss of correct interaction with mitotic microtubules, since it was observed that the KAP1-targeting constructs disrupted the kinetochore core structure assembled on the HAC (figure 26-27). Although direct evidence was not provided, I could rule out an effect of the targeting on the HAC's replication. In fact, the presence of double YFP spots, i.e. the two

duplicated HAC sister chromatids, in transfected mitotic cells gave evidence that a replication of the HAC had occurred.

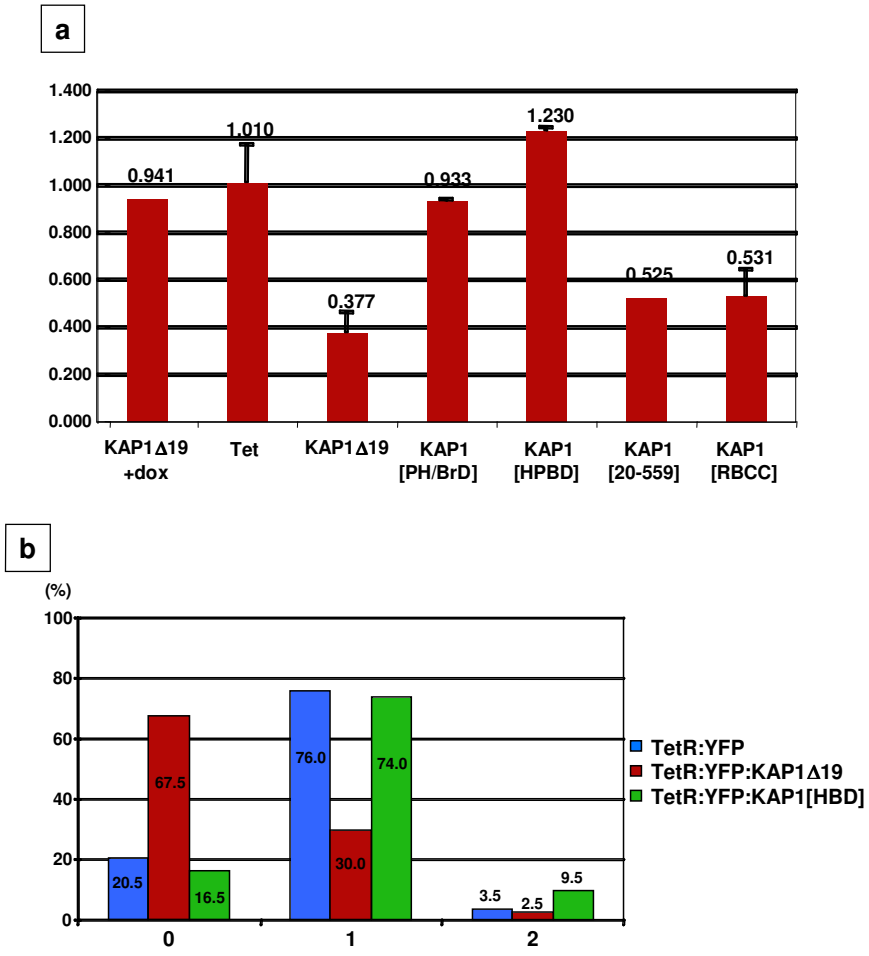


Figure 30. HAC retention in transfected cells.

(a) qPCR on genomic DNA prepared from cells transfected with the indicated constructs and cultured for 9 days. The graph shows the template amount (average of tetO and Bsr sequences) relative to total amount of DNA. The values are relative to the control experiment TetR:YFP+doxycycline (value 1.000). **(b)** Cytological analysis of HAC retention in cells transfected with the indicated constructs, selected with the drug puromycin and cultured for 12 days. FISH was used to quantify the fraction (%) of nuclei retaining the HAC (0, 1, 2).

3.8 HPIα recruitment and CENP-C association

The analysis of association of CENP-C and CENP-H to the HAC upon targeting showed that the domains of KAP1 affect differently the association of the two pre-kinetochore components. In fact, only the RBCC region, individually or in combination with the HPI-binding sequence, but not the other domains of the protein, appeared to efficiently affect the recruitment of CENP-C (Figure 27g).

To better clarify the role of HPI-recruitment to the KAP1 disrupting activity of the kinetochore assembly, I co-stained cells with both anti-HPIα and anti-CENP-C antibodies. The hypothesis is that if the association of HPIα was not compatible with centromere activity, CENP-C would not co-localize with HACs enriched for HPIα. KAP1Δ19 and KAP[HBD] constructs were transfected together with TetR:YFP into 1C7 cells, which were fixed 48 hours post-transfection for immunostaining. The binding of TetR:YFP to the HAC was not associated with detectable HPIα staining (Figure 31a). However, a strong signal for HPIα was observed in 76% of the cells transfected with KAP1Δ19 (Figure 31b, c). Reproducibly, 53% of the HACs positive for HPIα did not have detectable CENP-C associated (Figure 31b'' and 31c). In the remaining 23% of the HACs where some signal could be detected, the quantification revealed a decrease of the amount of CENP-C compared to the amount associated after targeting with TetR:YFP or the C-terminus construct KAP1[PH/BrD] (Figure 31d). Conversely, all HPIα-negative HACs were positive for CENP-C (Figure 31c).

In contrast to what we expected, targeting of the HPI-binding region of KAP1 recruited HPIα only in 29% of the HACs analyzed (Figure 31c). Of these, only 16.5% had CENP-C signal associated, albeit at a reduced level (not shown).

This analysis suggests that recruitment of HPIα to the synthetic alphoid^{tetO} DNA on the HAC is not compatible with the association of inner kinetochore component CENP-C. Furthermore, the inability to efficiently recruit HPIα of the KAP[HBD] construct, could account for the absence of an effect on either

CENP-C recruitment staining (Figure 27g) and HAC mitotic stability by this construct (Figure 30).

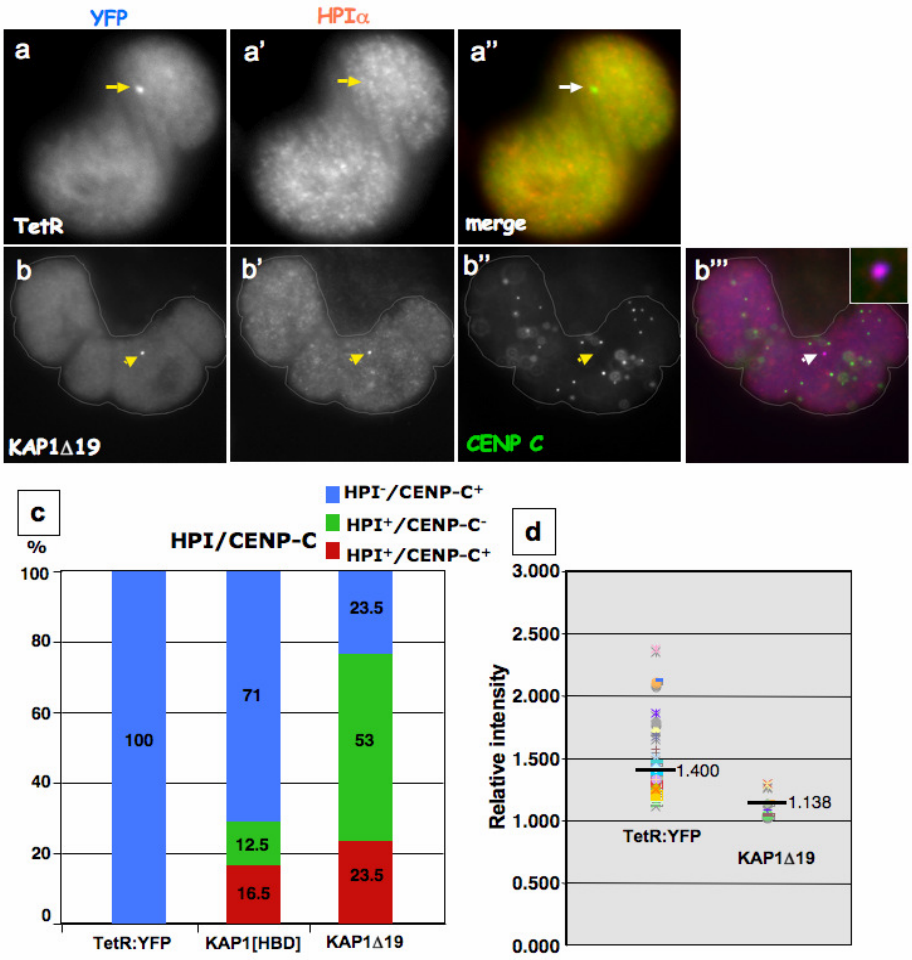


Figure 31. Co-recruitment of HPIα and CENP-C.

Cells were transfected with TetR:YFP and TetR:YFP:KAP1 constructs and fixed at 96 hours for staining with anti-CENP-C (b'') and anti-HPIa (a'-b') antibodies. **(c)** Quantification of the fraction of targeted HACs (%) showing presence or absence of co-association of HPIa and CENP-C (+/+, -/+, +/-). **(d)** Quantification of CENP-C signal associated with the HAC after targeting with the indicated constructs. Cells were transfected and fixed 96 hours post-transfection for staining and only the HACs with some remaining staining were measured. The HAC-associated CENP-C signal was normalized for the background. Only nuclei with similar expression (i.e. background signal) were selected.

4. Effect of HPIα targeting to the HAC in the 1C7 cell line

Targeting the Heterochromatin Protein 1 (HPIα) to the alphoid^{tetO} HAC in the AB2.2.18.21 cell line (Chapter III) was able to efficiently inactivate the HAC's synthetic kinetochore.

To test whether HPIα could destabilize the alphoid^{tetO} HAC also in a different cell line, I transfected 1C7 cells with TetR:YFP and TetR:YFP:HPIα and stained after 48 hours with an antibody against CENP-C. Confirming the results obtained with the HT1080 cell line, TetR:YFP:HPIα disrupted the association of CENP-C with the HAC in 45% of the cells expressing this construct (Figure 32). In parallel controls, no effect could be observed after targeting TetR:YFP.

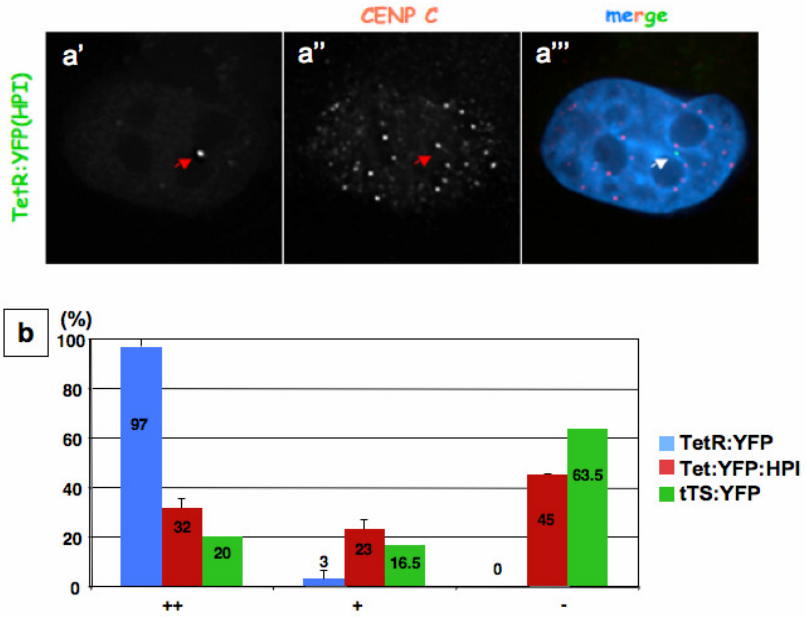


Figure 32. Targeting of TetR:YFP:HPI

1C7 cells were transfected with TetR:YFP, TetR:YFP:HPI and tTS:YFP, and stained for CENP-C 48 hours post-transfection. **(a)** HPI-targeted HAC loses the association of CENP-C. **(b)** quantification of the fraction (%) of cells that have strong (++) weak (+) or no (-) staining for CENP-C.

5 Targeting of histone methyltransferase Ezh2

5.1 Introduction

It has been proposed that KAP1 functions as a scaffold protein, recruiting chromatin modifiers at loci that need to be transcriptionally silenced (Schultz, 2000). This mechanism is most probably mediated by the formation of heterochromatin at the specific targeting site. The inactivation of the centromeric functionality of the alphoid^{tetO} DNA has been mapped to the NH2-terminus RBCC and the HPI-binding regions of KAP1. The first region is involved in binding the KRAB-domain of the Kruppel-type of transcriptional repressors; the second, to binding HPIα and therefore mediating the transcriptional silencing (Lechner et al., 2000; Schultz et al., 2001). To understand whether the formation of silent chromatin per se is sufficient to inactivate the alphoid DNA, I set out to target the alphoid^{tetO} HAC with a different type of chromatin conformation that is associated to gene silencing.

The homeotic box (*Hox*) is a chromosome locus in *Drosophila* that is characterized by a spatial pattern of expression, established during early development and subsequently maintained during mitotic cell divisions (reviewed in Cao and Zhang, 2004). This mechanism has been referred to as "cellular memory". Two groups of transcriptional regulators are involved in this regulation: the polycomb (PcG) and the trithorax (trxG) groups. PcG proteins are transcriptional repressors, whereas trxG proteins are transcriptional activators. In *Drosophila,* PcG proteins function in two distinct complexes: the Polycomb repressive complex 1 (PCR1) and the Extra sex combs and Enhancer of Zeste (ESC-E[Z]) complex (also know as PRC2 or EED-EZH2 in mammals). PRC2 contains an intrinsic methyltransferase activity for lysine 27 of histone H3, which is required for the silencing activity (Muller et al., 2002). In fact, H3-K27 methylation acts as an anchor for the binding of the PRC1 complex, which may inhibit chromatin remodelling, leading to transcriptional silencing (Cao et al., 2002; Shao et al., 1999). This H3K27me3-mediated mechanism was chosen in order to nucleate another type of gene silencing in the HAC alphoid^{tetO} region.

5.2 EZH2-targeting and CENP-C recruitment

To trigger H3K27me-associated chromatin modification on the alphoid^{tetO} HAC, I cloned from human cDNA the coding sequence of EZH2, which is the mammalian homolog of *Drosophila* histone methyltransferase *Enhancer of zeste,* a component of the PRC2 complex (Cao et al., 2002; Czermin et al., 2002). To construct the HAC-targeting construct, I fused EZH2 to the C-terminus of TetR:YFP, on the pEYFP-C1 variant containing the resistance gene for the drug puromycin.

To test whether the TetR:YFP:EZH2 construct retained enzymatic activity on histone H3, I stained cells transfected with TetR:YFP:EZH2, and control TetR:YFP, with an antibody for trimethylated K27 of histone H3 (H3K27me3). The staining showed enrichment of H3K27me3 only on the HAC targeted with EZH2 (Figure 33a), and not after targeting with TetR:YFP (not shown). Furthermore, since the PRC1 complex binds to methylated K27 on histone H3, I analyzed the recruitment of the PRC1 component RING1 to the alphoid^{tetO} HAC following the targeting of EZH2. Indeed, a clear signal for RING1 co-localized with TetR:YFP:EZH2 (Figure 33b) and not with TetR:YFP (not shown).

To analyze the effect of EZH2 activity on the binding of pre-kinetochore complexes to the modified alphoid^{tetO}, I transfected 1C7 cells with TetR:YFP:EZH2 and TetR:YFP, fixed them at 96 hours and stained for CENP-A and CENP-C. The quantification of the number of nuclei positive or not for CENP-A and/or CENP-C showed that only 3% of the HACs positive for CENP-A lacked detectable CENP-C staining following EZH2 targeting (Figure 33c-d). Furthermore, no HACs were found that had lost both CENP-A/C stainings (Figure 33d), and CENP-C could be found associated to HACs with clear enrichment for H3K27me3 (Figure 33a'') or RING1 (Figure 33b''). However, the analysis of the amount of CENP-C signal on the EZH2-bound HACs revealed that this was on average slightly less than for the control TetR:YFP-bound HACs (Figure 33e).

These results showed that it is possible to bind an enzymatically active H3K27 methyltransferase to the alphoid^{tetO} HAC, and modify the underlying DNA nucleosomes. This modification was able to function as a binding substrate for the polycomb PRC1 complex. This type of modification had a slight effect on the amount of bound inner kinetochore CENP-C, but this effect was much less severe compared to targeting of the KAP1-mediated heterochromatic gene silencing.

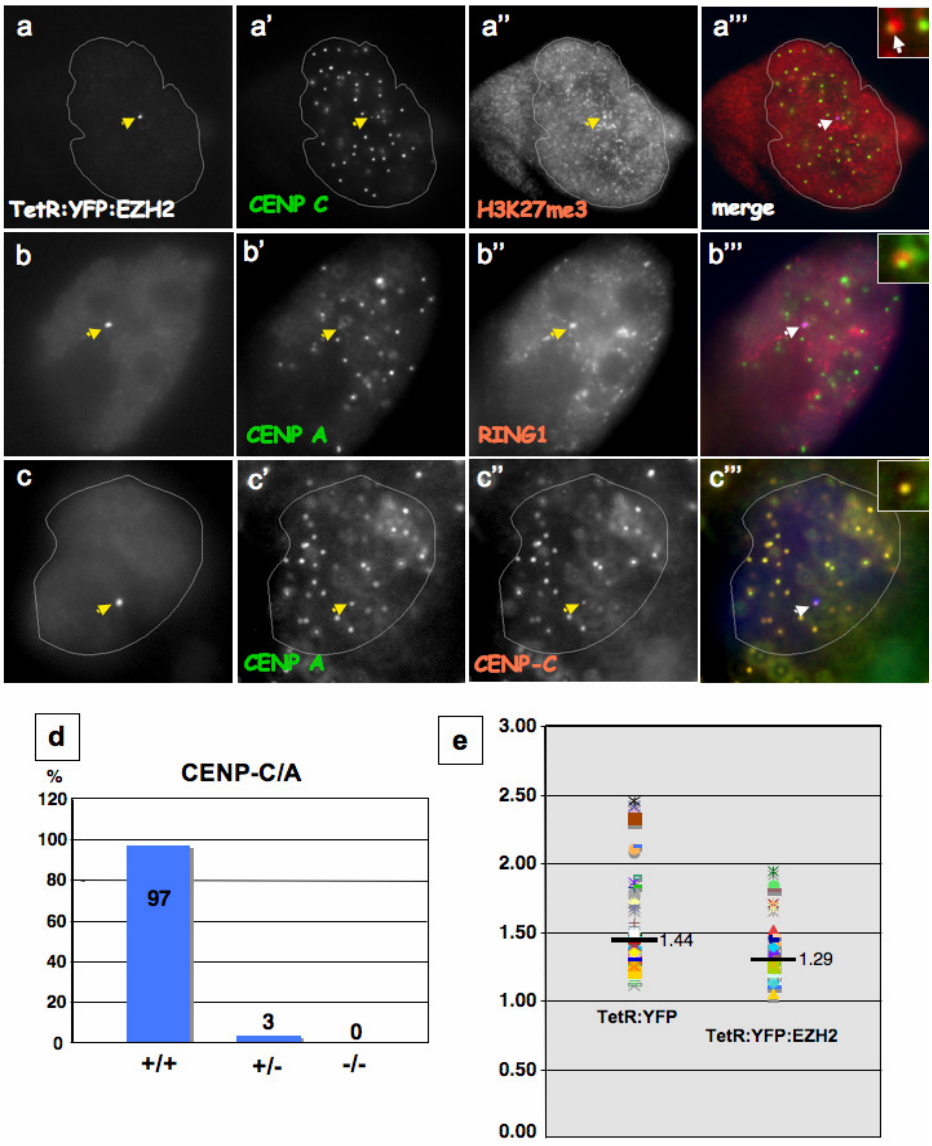


Figure 33. Targeting of TetR:YFP:EZH2 to the alphoid^{tetO}HAC
1C7 cells were transfected with TetR:YFP:EZH2 and fixed 96 hours post-transfection for staining with antibodies against H3K27me3 (a), RING1 (b), kinetochore proteins CENP-A (c'') and CENP-C (c'''). **(d)** Frequency (%) of targeted HACs that have lost both CENP-C and CENP-A (-/-), only CENP-C (+/-) or co-stained with both antibodies (+/+). **(e)** Quantification of the amount of CENP-C associated to the HAC after targeting with TetR:YFP and TetR:YFP:EZH2.

6. Conclusions

Thus far, all human artificial chromosomes characterized were obtained in human fibrosarcoma HT1080 cell lines. The reason why this is the only human cell line that shows HAC formation ability is unknown. The HT1080, AB2.2.18.21 cell-line used (Chapter III) to manipulate *in vivo* the alphoid^{tetO} HAC chromatin had two limitations: low transfection efficiency with conventional methods and cells had long doubling time. These two characteristics were not compatible with using AB2.2.18.21 cells for a proteomic study of the active versus invactive alphoid^{tetO} HAC. In fact, a proteomic analysis requires a large number of cells to successfully apply biochemical approchaches to characterize protein complements. Thus, to obtain a human cell line with the synthetic HAC that is more suitable for proteomic studies, and to verify whether the effects observed with tTS targeting in AB2.2.18.21 cells are cell-line specific, I fused this cell line containing the alphoid^{tetO} HAC to HeLa cells, and selected cell clones that showed a HeLa phenotype.

The alphoid^{tetO} HAC was highly stable also in HeLa-hybrid cells (1C7) and the underlying tetO sequences were accessible to TetR-targeting constructs. Also in 1C7 cells, the targeting of tTS destabilized the HAC kinetochore. To understand the mechanism of HAC kinetochore inactivation mediated by tTS, I targeted to the synthetic HAC the transcriptional co-repressor KAP1, which is thought to mediate tTS activity.

KAP1Δ19 (lacking the first 19 amino acids) severely disrupted the HAC kinetochore and impaired its mitotic stability. Individually, all KAP1 subdomains appeared to disrupt the association of CENP-H to the alphoid^{tetO} DNA, but not all were as efficient to disrupt CENP-C association. In fact, the KAP1 N terminal RBCC region alone was able to disrupt CENP-C recruitment to the HAC kinetochore and increase HAC loss rate from the population of transfected cells.

Intriguingly, a large proportion of HACs targeted with KAP1Δ19 that had lost CENP-C, still recruited the centromere-specific histone H3 variant CENP-A. This result suggests that the disruption of CENP-A assembly into HAC alphoid^{tetO} DNA might be consequent to the loss from the HAC of constitutive inner kinetochore proteins.

In agreement with what is expected from the loss of the CENP-H/I complex from the inner kinetochore, KAP1Δ19-targeted HAC often lacked Ndc80/HEC1 at the outer kinetochore, while CENP-A or CENP-C were often still present at the inner kinetochore. This resulted in mitotic misalignment and lack of inter-kinetochore tension, which might preceed defective mitotic segregation and loss of the HAC from the divided cell.

In addition to the HAC-inactivating activity of the N-terminal RBCC motifs of KAP1, another effect might be linked to the recruitment of HPIα. In fact, the association of CENP-C was inversely correlated with the appearance of HPIα on the KAP1Δ19-targeted HAC. In support of this, the targeting of HPIα to the alphoid^{tetO} DNA also delocalized inner kinetochore proteins.

The inactivation of HAC kinetochore seemed to be linked to HPIα-based heterochromatin nucleation. In fact, although the targeting of the histone methyltransferase EZH2 enriched the synthetic HAC alphoid of trimethyl-Lysine 27 of histone H3, which is a marker for heterochromatin, this modification did not significantly affect pre-kinetochore assembly.

V. Discussion

Construction of a Human Artificial Chromosome from synthetic alphoid DNA

The construction of artificial chromosome vectors in yeast allowed the first definition of the chromosomal elements that are necessary for the stable perpetuation of chromosomes through cell generations, (Murray and Szostak, 1983). However, in humans the construction of artificial chromosomes was hindered by a lack of information on the corresponding chromosomal elements in mammals. Once light was shed on the nature of these arrays of repeated sequences (Willard, 1996), another great obstacle for HAC construction was the inability to clone large, stable fragments of repeated DNA (Neil et al., 1990). The development of new cloning strategies allowed the construction of the first generations of HACs from known, cloned DNA sequences (Ikeno et al., 1998).

We succeeded to construct, for the first time, a Human Artificial Chromosome from a new type of synthetic alphoid DNA array. This new type of α-satellite DNA was designed to contain an array of sequences that are recognized and bound by recombinant proteins, and to allow *in vitro* and *in vivo* manipulation of *de novo* constructed HACs. This alphoid array contained CENP-B boxes in half of its monomers, whilst in the other half, tetO sequences were inserted in their place. This type of array was much less efficient. However, once the alphoid^{tetO} HAC was formed, it showed mitotic stability similar to HACs constructed from natural alphoid DNA from Chromosomes 17 and 21. The alphoid^{tetO} HAC was stably retained for many generations in the HT1080 cells where it was initially obtained. In addition,

after transfer into a HeLa-fusion cell line, the alphoid^{tetO} HAC retained high stability in cycling cells, successfully completing cycles of replication and mitotic segregation. Thus far, this is the first analysis of human artificial chromosome behaviour in a human cell line different from HT1080.

The distribution and enrichments of specific histone modifications associated with the HAC alphoid^{tetO} array in HT1080 cells appeared overall similar to those associated with HACs obtained from endogenous alphoid DNA from Chromosome 21 and to endogenous centromeres. The amount of the centromere-specific histone H3 variant CENP-A was similar to that found at endogenous centromeric repeats. However, the alphoid^{tetO} array of the HAC in HT1080 cells appeared to contain more of the H3K4me2 modification. Since this modification has been classically associated to a transcriptionally "open" DNA conformation, this result suggests that the chromatin of our newly constructed HAC might be more transcriptionally competent than human centromeres.

Remarkably, the array of tetO sequences was embedded into the stable alphoid^{tetO} HAC kinetochore, and yet was accessible to the binding of TetR proteins. I have shown that it was possible to tether TetR-linked proteins specifically to the alphoid^{tetO} DNA array. The binding of YFP-fused TetR proteins did not disrupt the replication of the HAC, as it was possible to observe pairs of YFP dots – replicated HAC sister chromatids - aligning on a metaphase plate, and segregating properly in anaphase and telophase.

This new type of HAC, containing a synthetic alphoid^{tetO} array of sequences, represents a second generation of human artificial chromosomes, which allow *in vitro* and *in vivo* manipulation. The possibility to manipulate at will a synthetic stable chromosome, carrying genes or sequences of interest, could open the door to new exciting biomedical applications, such as gene transfer and human gene therapy.

Inactivation of a human synthetic centromere by targeting of transcriptional activators

The exact nature of centromeric chromatin is still obscure. In fact, recently it has been shown that not only a marker of "open" chromatin (H3K4me2) is present at centromeric alphoid DNA (Willard, 1996; Sullivan and Karpen, 2004; Lam et al., 2006), but also H3K9me3, a modification usually associated to heterochromatin, was found enriched at sub-domains of these sequences (Nakashima et al., 2005; Lam et al., 2006).

The accessibility of the array of tetO sequences present in the HAC synthetic alphoid DNA to the targeting of TetR proteins, allowed the design of strategies to modify the chromatin of the alphoid^{tetO} HAC. The aim of the proposed studies was to define what type of chromatin is suitable to sustain centromere functions.

The first approach used for altering the HAC alphoid^{tetO} chromatin was the targeting of the transcriptional activator tTA which consists of a combination of the transcription activation domain of Herpes Simplex virus VP16 and a class B *E. coli* TetRepressor sequence. This construct has been widely used to drive transcription from selected genes in all types of organisms. Importantly, the fusion of tTA to the Yellow Fluorescent Protein (YFP) did not impair its ability to activate transcription from the targeted alphoid^{tetO} sequences. Targeting tTA to the alphoid^{tetO} HAC disrupted the binding of constitutive kinetochore CENP- proteins, which led to defects in mitotic segregation and inclusion of the HAC in small cytoplasmic structures (nano-nuclei). However, the tTA appeared to destabilize the HAC in some cells but not in others (mosaic effect), resulting in an overall much weaker effect compared to tTS targeting (see next section). This phenomenon has a number of possible explanations.

For example, CENP-A chromatin may tolerate a degree of chromatin "opening" and transcriptional activity while retaining kinetochore activity. This model is supported by the evidence that the chromatin of the alphoid^{tetO} HAC,

in the AB2.2.18.21 cell line, is enriched in the H3K4me2 histone modification, compared to endogenous alphoid sequences. In addition, a basal (low) level of transcriptional activity from the alphoid^{tetO} array could be physiologically detected. The presence of actively transcribed genes within rice centromeres (Nagaki et al., 2004) and human neocentromeres (Saffery et al., 2003), plus the detection of low level transcription of alphoid sequences from human chromosomes transferred in DT40 cells (Fukagawa et al., 2004), suggest that in many cases, functional kinetochores can contain "open" chromatin. It is possible that low levels of transcription are tolerated within kinetochore chromatin, however high level transcription triggered by a stochastic event occurring in a subset of cells expressing the tTA may disrupt kinetochore function. This effect might be mediated by the binding of tTA to other general transcription factors including the TATA-binding protein (TBP), TFIIB, and the SAGA histone acetylase complex (Hall and Struhl, 2002; Klein et al., 2003; Herrera and Triezenberg, 2004). The inactivation of a yeast kinetochore by bombardment with transcription (Hill and Bloom, 1987), also supports this model.

Kinetochore inactivation by heterochromatic gene silencing

The second approach used for altering the HAC alphoid^{tetO} chromatin was the targeting of chromatin modifiers that induce nucleation of heterochromatin at the targeted sequences. For this purpose, I obtained three different targeting constructs. The first was the transcriptional repressor tTS, a combination of a hybrid Class B/E TetR protein and a KRAB (Kruppel-Associated Box) repression domain of the transcriptional repressor Kid1 (reference). Secondly, since the KRAB-containing family of transcriptional repressors are thought to work by binding to the transcriptional co-repressor KAP1 (Kruppel-associated protein I), I also constructed a targeting construct consisting of a TetR:YFP fused to amino acids 20-835 of KAP1 (generously provided by Dr. D Schultz). Thirdly, it was decided to target HPIα, which has

been involved in the stabilization and spreading of heterochromatin (Smothers and Henikoff, 2000; Lechner et al., 2005).

In contrast to the mosaic effect observed with tTA targeting, transcriptional repressor and co-repressor tTS and KAP1 were significantly stronger disruptors of the centromeric function of the synthetic alphoid^{tetO} DNA of the HAC. This disruption was unlikely an effect of the passive binding of bulky chromatin remodelling complexes to the underlying alphoid sequences. The targeting of a variety of proteins to the alphoid^{tetO} HAC, including a mutated variant of tTS (tTS^{mut}), failed to show any inactivating effect. Noticeably, tTA and the C-terminus domains of KAP1 that also bind a spectrum of chromatin remodelling complexes (Schultz et al., 2001), had a much milder disrupting effect on HAC centromere function.

Two models might explain the HAC alphoid^{tetO} inactivation mediated by tTS and KAP1. The first possibility is that the nucleation of heterochromatin *per se* is incompatible with a functional centromere. The second possibility is that a basal level of transcription from centromeric sequences is necessary for preserving centromere activity and/or inheritance.

Although centromere heterochromatin has long been described as heterochromatic, recent reports argue against this model (reviewed in Schueler and Sullivan, 2006). For example, it has been observed that overexpressed CENP-A is not incorporated into heterochromatin (Vermaak et al., 2002). In addition, in *Drosophila,* heterochromatin can block neocentromere formation (Maggert and Karpen, 2001).

The targeting of tTS to the alphoid^{tetO} HAC caused a drop of the H3K4me2 and H3K4me3 histone modifications, which are markers for transcriptionally competent or active chromatin. The inability of the tTS^{mut}, which is a variant of tTS mutated within the KRAB domain, to inactivate the alphoid^{tetO} HAC suggested that tTS effects its action by recruiting KAP1. KAP1 is thought to silence transcription by nucleating heterochromatin at targeted chromosomal loci (Sripathy et al., 2006; Ayyanathan et al., 2003). In

fact, the targeting of KAP1 caused also a slight but significant change of the underlying DNA towards a “heterochromatic” state.

The type of heterochromatin that triggers the inactivation of the HAC alphoid DNA seems to be tightly associated with the recruitment of HPIα. The kinetochore disruption seemed to be inversely related to the recruitment of HPIα by KAP1 and its HPI-binding domain. Moreover, the targeting of HPIα itself to the alphoid^{tetO} HAC was able to efficiently disrupt kinetochore assembly on the HAC. Therefore, the centromere-killing activity of the tTS and KAP1 might rely, at least in part, on their ability to nucleate H3K9me3-type heterochromatin, which in turn acts as a binding substrate for HPIα, or possibly by directly recruiting HPIα to the underlying chromatin.

To better understand the role of the heterochromatin, I wanted to probe a different type of inactive chromatin for an effect on HAC kinetochore assembly. Another type of reported heterochromatin-mediated gene silencing involves the methylation of Lysine 27, instead of Lysine 9, on histone H3 (H3K27me3). This is mediated in mammals by EZH2 methyltransferase. H3K27me3-modified histones are then substrates for the binding of polycomb-group proteins that silence transcription, most probably by modifying the chromatin. To target the alphoid^{tetO} HAC with this type of chromatin modification, I fused EZH2 to TetR:YFP. The targeting of EZH2 caused a slight decrease in the amount of associated CENP-C, but the effect was much weaker compared to the targeting of KAP1 or HPIα. This result suggests that not all types of heterochromatin but specifically HPIα-associated heterochromatin is responsible for the inactivation of the synthetic alphoid^{tetO} array.

An alternative idea for the inactivating mechanism was suggested by the fact that tTS binding led to a rapid drop in the levels on the alphoid^{tetO} array of H3K4me2, which appeared to slightly precede the loss of CENP-A from the alphoid^{tetO} array (compare time points 7 and 14 days, Figure 21). This result suggests that the H3K4me2 modification *per se* might have an essential role in helping to maintain the structure of centromere chromatin.

A second possible mechanism for inactivation of the alphoid^{tetO} HAC involves the suppression of a basal transcriptional activity from the alphoid sequences. This model is supported by the fact that the targeting of just the N terminal KRAB-binding (RBCC) region of KAP1 is sufficient to effectively disrupt the alphoid^{tetO} HAC kinetochore. In addition, some indications on the role of transcriptional activity for centromere functionality came from the targeting of the histone methyltransferase EZH2. Although the ability of EZH2 to silence transcription was not tested, the appearance of H3 histones enriched in the K27me3 modification and the recruitment of the polycomb PRC1 complex both suggest that EZH2 nucleates silent chromatin at the targeted sequences. HAC kinetochore disruption by EZH2 was considerably milder than by tTS or KAP1. This suggests that not all types of chromatin-mediated gene silencing can inactivate alphoid repeats as kinetochore components. A more detailed analysis of the effects of the targeting of EZH2 on the HAC mitotic stability is required.

In conclusion, the tTS- and KAP1-mediated disruption of the assembly the alphoid^{tetO} HAC kinetochore might reside within their ability of nucleating HPIα-heterochromatin on the targeted alphoid sequences, but also partially on the inhibition of transcription or recruitment of additional transcriptional repressors.

VI. Conclusions

Centromeric chromatin or kinetochore structure: what comes first?

The targeting of a transcriptional repressor and a corepressor efficiently disrupted the binding of several inner and outer kinetochore proteins to the alphoid^{tetO} HAC. This kinetochore inactivating activity had the most severe effect on CENP-H. In fact, the loss of CENP-H binding to the alphoid^{tetO} array of the HAC was the most widespread and could readily be observed with all KAP1 domains. A reproducible loss of the constitutive inner kinetochore protein CENP-C was also observed, but to a lesser extent than for CENP-H and the effect was limited to the KAP1Δ19 or the N terminal part of the protein. This suggests that the CENP-H/I complex is the first to be delocalized from the kinetochore by these treatments. Since the CENP-H/I complex is required for the recruitment of CENP-C in chicken interphase cells (Nishihashi et al., 2002), the loss of CENP-H from the HAC kinetochore might subsequently result in the de-localization of CENP-C. Alternatively, CENP-H and CENP-C might be recruited by distinct pathways and their dissociation might not be correlated (Liu et al., 2006).

CENP-C is found upstream of at least 4 different branches of the recruitment of outer kinetochore proteins, including the HEC1/Ndc80 subunit hNuf2 (Liu et al., 2006). Therefore, a lack of sufficient CENP-C associated with the inner kinetochore might cause de-localization of outer kinetochore protein complexes that are required to establish functional attachments to spindle microtubules. As expected, the outer kinetochore protein HEC1 also did not localize to the inactivated alphoid^{tetO} HAC kinetochore lacking of CENP-C. However, to further define the mutual CENP-C and HEC1 localizations at the HAC kinetochore, the association of hMis12, which has been placed downstream of CENP-C (Liu et al., 2006), must be investigated.

Alternatively, it is possible that the disruption of CENP-H/I binding to the HAC kinetochore affects the localization of the HEC1/Ndc80 complex. In fact, it has been reported that hKNL1 and the CENP-H/I complex assist cooperatively the recruitment of HEC1/Ndc80 to the outer kinetochore (Cheeseman et al., 2008).

The data presented here show that the disrupted HAC kinetochore is not able to sustain functional microtubule attachments. This was deduced from the observations that the YFP-positive pairs of HACs failed to properly align on a metaphase plate, and the distance between sister chromatids was much reduced compared to controls.

Intriguingly, disruption of the kinetochore structure might precede the loss of centromeric chromatin, as defined by the presence of CENP-A-containing nucleosomes. Almost 40% of the HACs destabilized by KAP1 showed loss of CENP-H while CENP-A was retained. In addition, the targeting of the lesser efficient KAP1 HPI-binding (KAP1[HBD]) and PHD/Bromodomain (KAPI[PH/BrD]) constructs showed almost always loss of CENP-H but retention of CENP-A nucleosomes. More importantly, in 50% of the HACs inactivated by targeting KAP1Δ19 and its N-terminal domains, CENP-C was lost but a strong signal for CENP-A could still be detected. However, in the other 50% of the disrupted HACs, CENP-A and CENP-C were both de-localized from the inactive HAC kinetochore. Although CENP-A has been widely reported to be at the top of at least one main pathway for kinetochore assembly (reviewed in Cleveland, 2003), it has recently been suggested that inner kinetochore proteins may also mediate CENP-A localization. In chicken DT40 cells, CENP-H/I complex is required for the assembly of CENP-A at the centromere (Okada et al., 2006). In addition, also CENP-C has been reported to affect CENP-A incorporation at the centromere (Goshima et al., 2007). The data presented suggest that the loss of CENP-A chromatin might be caused by the loss of a constitutive pre-kinetochore complement. KAP1 binding might cause structural chromatin changes that impair the assembly of constitutive pre-kinetochore components onto the underlying alphoid^{tetO}

DNA, and this might be *per se* sufficient for impairing the faithful propagation of the alphoid^{tetO} centromere activity (Figure 34). This model is supported by the fact that newly synthesized CENP-A is incorporated into centromeres only from mitotic telophase, after kinetochores have been fully assembled (Jansen et al., 2007; Schuh et al, 2007). Despite of recent evidences (Schuh et al, 2007), it remains to be defined whether the establishment of inter-kinetochore tension by pulling forces in mitosis triggers epigenetic signals that are required for the faithful inheritance of centromeric activity (reviewed in Mellone and Allshire, 2003). In this scenario, the lack of microtubule attachment and inter-kinetochore tension on the inactivated HAC might affect the incorporation of CENP-A into the HAC synthetic alphoid array.

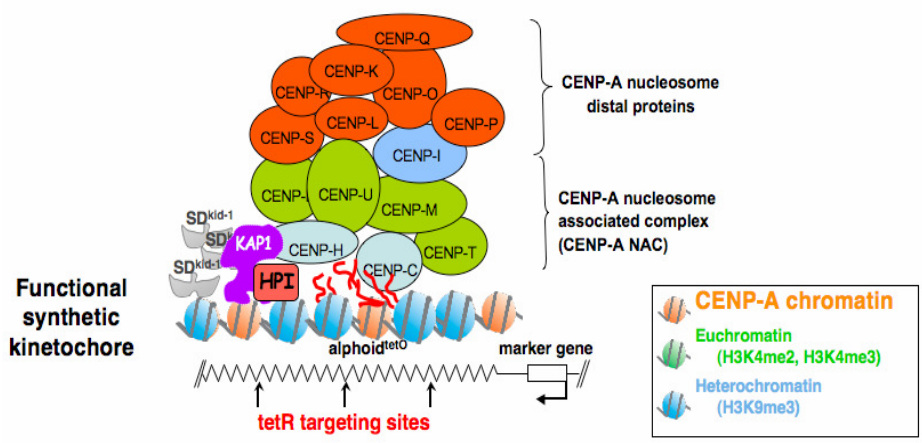


Figure 34. Model for the kinetochore inactivation by KAP1/HPIα

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VII. Appendix

1. List of figures.

2. List of tables